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EFFECTS OF CASTE ON THE CONSTITUTIVE AND INDUCED EXPRESSION OF GENES ASSOCIATED WITH IMMUNITY AND DETOXIFICATION IN FORMOSAN SUBTERRANEAN TERMITES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Entomology

by
Dawn M. Simms
B.S., Louisiana State University
May 2013

“You could give Aristotle a tutorial. And you could thrill him to the core of his being ... Such is the privilege of living after Newton, Darwin, Einstein, Planck, Watson, Crick and their colleagues.”

- Richard Dawkins

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Abstract

Formosan subterranean termites (FSTs) live in dense populations and nest conditions that are conducive to microbial growth. Furthermore, termites are exposed to potential toxic substances in the soil and in their lignocellulose diet. These characteristics, in combination with the specialized caste system of FSTs, make this termite a valuable model for studying insect immunity and detoxification mechanisms. Since workers forage for food, feed their nest mates and care for the brood, they are more likely than soldiers to encounter pathogens and/or toxins. They must, therefore, be better able to defend against these challenges. This study tested the hypothesis that genes associated with immunity and/or detoxification via xenobiotic metabolism in FST have higher constitutive expression levels and/or are more readily inducible in workers than in soldiers. FST workers and soldiers were challenged either by bacterial injection or by no-choice feeding with a sublethal concentration (0.5%) of Phenobarbital. Constitutive and induced expression of 5 putative immune response and 5 detoxification genes was measured via quantitative real time qRT-PCR and compared within and between 1) colonies, 2) treatment groups and 3) castes via non-parametric, multivariate analysis of variance. Of the 10 total target genes, none were found to be differentially expressed as an effect of colony. However, treatment type had a significant ($P < 0.05$) effect on the expression of each of the 8 target genes that were inducible. Caste effects on expression levels were significant in 6 of the 8 inducible target genes. In many cases, target genes were only inducible in the worker caste. Overall, constitutive and induced expression of target genes was significantly higher in workers than in soldiers.

Chapter 1: Introduction and Literature Review

1.1 Formosan Subterranean Termites

1.1.1 General Characteristics

Coptotermes formosanus (Blattodea: Rhinotermitidae), common name: Formosan subterranean termite (FST), colonies live in underground nests, hidden from natural predators and humans. An FST colony consists of one (or more) reproductive pair(s) and their offspring, which can be over a million in number (Shellman-Reeve 1999, Vargo et al. 2003a, Vargo et al. 2003b, Husseneder et al. 2005, Vargo et al. 2006, Husseneder and Simms 2008). Although termites are sometimes referred to as "white ants", they are actually much more closely related to cockroaches and mantids than they are to ants (Klass and Meier 2006, Inward et al. 2007, Legendre et al. 2008). Like ants, however, the FST is eusocial, which means that colony members share resources, cooperatively care for the brood and have a clearly defined caste system with a division of labor (Suiter et al. 2002).

1.1.2 Life Cycle and Colony Structure

FST colonies originate from a pair of alates (winged adults) that participate in mating flights during the swarming season (late April to June in Louisiana: Simms and Husseneder 2008). Mass swarms contain alates from several colonies, which may each release alates numbering in the thousands in a single night (Su and Scheffrahn 1987). The swarms operate as a mechanism of partner selection (Raina et al. 2003, Park et al. 2004, Husseneder et al. 2008), after which, the mated pairs move underground, and the queen begins to lay eggs. The founding reproductives are called the primary king and queen. They are monogamous and mate for life, producing millions of offspring during their lifetime (Shellman-Reeve 1999, Vargo et al. 2003a,

Vargo et al. 2003b, Husseneder et al. 2005, Vargo et al. 2006). The first offspring of the new colony (larvae) are dependent on parental fat reserves for nourishment (Shellman-Reeve 1999). Only after foragers (workers) develop does the colony begin to grow and mature. A "mature" colony is one that begins to produce alates; the time frame to reach maturity ranges from 4 to 8 years after colony foundation (Huang and Chen 1984, Fei and Henderson 2003).

The FST life cycle is plastic, that is, it changes to accommodate the needs of the colony. This plasticity is thought to be a factor in the success of FST as an invasive species (Vargo et al. 2003a, Vargo et al. 2003b, Husseneder et al. 2005, Vargo et al. 2006, Husseneder et al. 2011). When the primary king and queen die, they are replaced by secondary kings and queens, formed from nymphal offspring of the founding reproductives (Vargo et al. 2003a, Vargo et al.2003b, Husseneder et al. 2005, Vargo et al. 2006). This is what is known as an extended family colony structure (multiple, secondary reproductive pairs), as opposed to a simple family colony structure (single pair of primary reproductives). The worker caste is itself an example of the plasticity of the FST life cycle. Workers are non-reproductive immatures that forage for and distribute food throughout the colony, build the nest and interconnected tunnels and care for the brood (King and Spink 1974, Su and Tamashiro 1987). However, environmental factors, e.g. hormonal titers (Liu et al. 2005) or colony density (Mao and Henderson 2010) can trigger workers to become tertiary reproductive or soldiers. FST soldiers are reproductively sterile, are unable to feed (due to their enlarged mandibles) and are primarily responsible for defending the colony from invaders.

1.1.3 Invasiveness and Economic Impact

The FST was first described from specimens found in Taiwan, formally the island of Formosa (Shiraki, 1909) and has become one of the most important and aggressively invasive pest species worldwide (Global Invasive Species Database 2013). The first known FST invasion, outside of its native range, was in Japan in the early 17th century (Vargo et al. 2003, Husseneder et al. 2011). It is likely that the FST was transported to Hawaii from China via the sandalwood trade (Yates and Tamashiro 1990, Husseneder et al. 2011), possibly in the late 19th century (Su and Tamashiro 1987, Yates and Tamashiro1990). It is thought to have first come to the continental U.S. by way of military cargo ships, following the 2nd World War (Spink 1967). The first FST specimen identified in the continental U.S. (Charleston, South Carolina) occurred in 1957 (Chambers et al. 1988). It now inhabits 14 Chinese provinces (Wang et al. 2002), much of the Pacific Rim, including Japan and Hawaii (Su and Tamashiro 1987) and at least 10 of the continental United States (Woodson et al. 2001).

Annual costs of FST damage and management efforts exceed $1 billion in the United States today (Culliney and Grace 2000, Pimentel et al. 2005). In Louisiana, economic losses due to FST damage and control efforts are estimated at over $500 million per year (Baldauf 2000, Morgan, 2005). In addition to the destruction the FST causes to manmade wooden structures, the termite feeds on at least 24 plant species in Louisiana, including living tree crops (Messenger and Su 2005). FST feeds primarily on the heartwood of trees, reducing structural integrity and causing tree collapse. FSTs are also known to chew through electrical and telephone cables and cause damage to other materials in their search for food and moisture (Lai et al. 1983, Su and Tamashiro 1987, Felix and Henderson 1995, Lax and Osbrink 2003).

1.2 Current FST Management Practices

1.2.1 Conventional Chemical Treatments

USDA approved chemical treatments for FST include hexaflumuron and noviflumuron (Sentricon®) baits, as well as fipronil (Termidor®, BASF Corp.) and imidacloprid (Premise®, Bayer) barrier sprays. These treatments, when used according to label, are effective at eliminating subterranean termite colonies (hexaflumuron: Sajap et al.1999, Vargo et al. 2003, noviflumuron: Husseneder et al. 2007, fipronil: Potter and Hillary 2003, imidacloprid: Potter and Hillary 2003). Hexaflumuron [((N-3,5-dichloro-4-1,1,2,2-tetrafluoroethoxy-phenyl)amino)carbonyl-2,6-difluorobenzamide)] and noviflumuron [(((N-3,5-dichloro-2-fluoro-4-1,1,2,3,3,3-hexafluoro-propoxy-phenyl)amino)carbonyl-2,6-difluorobenzamide)] are insect growth regulator insecticides. The mode of action is inhibition of chitin synthesis, which is necessary for exoskeletal growth (Dow AgroSciences 2004, 2009). The chemical composition of these insecticides allows them to bind strongly to soil with low water solubility, which indicates low leaching potential (Dow Chemical Company 2011a, 2011b). However, hexaflumuron and noviflumuron have high potentials for environmental bioaccumulation and are toxic to aquatic invertebrates (U.S. EPA Office of Pesticide Programs 2003).

Fipronil [(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)-4-trifluoromethyl sulfinylpyrazole-3-carbonitrile] acts to disrupt GABA-gated chloride channels in the central nervous system, which prevents chloride ion uptake and causes continuous firing of neural synapses (Cole et al. 1993, Ratra and Casida 2001, Boyd et al. 2002). Fipronil is an irritant to humans, a possible carcinogen and is toxic orally, dermally or when inhaled, and its degradative products are also toxic to some birds, aquatic invertebrates, terrestrial invertebrates and mammals (Jackson et al. 2009).

Imidacloprid [[1-(6-chloro-3-3pyridyl-methyl)-N-nitro-imidazolidin-2-ylideneamine] is a neonicotinoid spray treatment. Imidacloprid acts at the postsynaptic nicotinic acetylcholine receptors of insects (Matsuda et al. 2001), which results in paralysis and/or death (Boyd et al. 2002). This chemical has been shown to leach into soil (Boyd et al. 2002, Jemec et al. 2007) and may contaminate nearby groundwater reserves (Boyd et al. 2002). Imidacloprid is harmful to several non-target and beneficial insects, including honeybees, predatory ground beetles and parasitoid wasps (Fossen 2006).

1.2.2 Biological Control Tactics

Microbial insecticides have been regarded as promising agents of biological control of FST and other soil-dwelling insect pests, as they are easy to produce, are non-deleterious to non-targets and do not require EPA registration (Kaya and Gaugler 1993, Kaya and Gaugler 1993). Four nematode families (Mermithidae, Allantonematidae, Steinernematidae and Heterorhabditidae: Popiel and Hominick 1992) have been successfully used in biological control programs, targeting invasive insect species; however, studies of nematodes management of field populations of termites have been disappointing (Culliney and Grace 2000). This is because the termites actively avoid sites that have been inoculated with nematodes (Culliney and Grace 2000). Furthermore, nematode coverage is limited by soil properties, physical barriers, abiotic factors, and competition with other soil-dwelling organisms (Gaugler 1988, Kaya 1990, Popiel and Hominick 1992, Chouvenc et al. 2011).

Endoparasitic fungi may be a potential biological control agent for controlling termites; however, very few fungal pathogens have been sufficiently researched for this purpose (Culliney and Grace 2000). Of the fungi that have been thoroughly investigated, *Metarhizium anisopliae* and *Beauveria bassiana* have been shown to cause rapid death upon ingestion (Zoberi 1990), and termite avoidance of fungal baits was less extensive than that seen for nematode baits (Boyd et al. 2002). Spread of fungal biological control agents throughout an FST colony may be limited, however, since the termites remove and/ or bury individuals killed by the fungus (Logan et al. 1990, Rath 2000, Chouvenc and Su 2010, Chouvenc et al. 2011, Chouvenc and Su 2012), thus eliminating horizontal endoparasitic transfer effects (Boyd et al. 2002, Chouvenc and Su 2010, Chouvenc et al. 2011, Chouvenc and Su 2012).

Biological control tactics employing entomopathogenic bacteria (e.g. *Serratia marcescens*, *Pseudomonas fluorescence*, *Staphylococous aureus*, and *Bacillus thuringiensis*) are impractical at best, since they have been unable to fully penetrate the FST nest (i.e. reach the reproductives and/or spread to brood chambers) and eliminate the entire colony (Culliney and Grace 2000, Boyd et al. 2002), which must be achieved in order to prevent reinfestation. Chouvenc and Su (2012) reviewed the literature comprising 50 years of research into the biological control of termites, and the shortcomings of said research. Of particular significance is the underlying lack of knowledge of termite biology and the synergistic defense mechanisms of subterranean termite colonies (Chouvenc et al. 2011). Inhibiting defense mechanisms, i.e. innate immunity signaling pathways and/or detoxification enzymes, could render termites more susceptible to infection (Boucias et al., 1996, Connick et al. 2001, Bulmer et al. 2009) and/or traditional chemical treatments; however, this field of research is yet to be fully developed or tested in the field (Chouvenc et al. 2011, Chouvenc and Su 2012).

1.2.3 Need for Improved Treatment Methods

Laboratory studies of biological control tactics have given promising results (Culliney and Grace 2000); however, these methods have not yet been shown to consistently control FST colonies in the field (Logan et al. 1990, Su and Scheffrahn 1998, Culliney and Grace 2000, Rath 2000, Chouvenc and Su 2010, Chouvenc et al. 2011, Chouvenc and Su 2012). Conventional termite treatments, though successful at eliminating FST colonies (Su and Scheffrahn 1996, Sajap et al.1999, Vargo et al. 2003, Su et al. 2004, Ring et al. 2006, Husseneder et al. 2007, Potter and Hillary 2003), rely heavily on chemical insecticides. While it may never be possible to eliminate the use of chemical treatments, it is critical that research continues to develop new types of termite treatments, which are not only effective, but also environmentally friendly (Lax and Osbrink 2003). Humans and termites live in close contact in the urban landscape, and public health concerns about adverse effects of chemical treatments are increasing (Rosengaus et al. 1999, Boyd et al. 2002). Although newer chemicals are less hazardous than most of the older chemical treatments (e.g. DDT, organochlorines), they are still hazardous, especially when misused (Rosengaus et al. 1999). The public is routinely exposed to reports of contaminated drinking water, fish kills, bioaccumulation and outbreaks of secondary pests, all of which result from widespread use of insecticides (Boyd et al. 2002). Therefore, it is important to develop new FST control technologies that decrease the amount of chemical insecticides necessary to reduce 1) termite damage, 2) further spread of invasive termites and 3) the costs for FST control.

1.3 Project Focus

1.3.1 Innate Immunity in Insects

The innate immune system operates to limit infection after exposure to microbes (Irving et al. 2001). The process begins with recognition of "non-self" antigens and ends with encapsulation, phagocytosis or otherwise destruction of foreign microbes (i.e. cell lysis or disruption of cell function). Invertebrates, including insects, have innate immune systems that are in many ways analogous to that of vertebrates, and are indeed thought to be precursors to the vertebrate innate immune system (Beck and Habicht 1996, Sompayrac 2012). Innate immunity has been conserved, for example, in *D. melanogaster* and in mammals (Irving et al. 2001). While insects lack macrophages (which are present in vertebrate animals), they have analogous molecules (e.g. coelomocytes) that similarly function to engulf and remove pathogens (Beck and Habicht 1996, Tsakas and Marmaras 2010, Sompayrac 2012). Also, while insects lack antibodies, they have pattern recognition cells (e.g. hemocytes) and molecules (e.g. immunoglobulin-like proteins) whose receptors bind to antigens, e.g. lipopolysaccharides and peptidoglycans, comprising cell membranes of foreign microbes (Beck and Habicht 1996, Rantala and Roff 2007, Bechkage 2008, Tsakas and Marmaras 2010, Sompayrac 2012).

Pathogen pattern recognition proteins (PRPs) are mainly synthesized in insects via fat bodies, hemocytes or epithelial cells, bind to the surface of pathogen associated molecular patterns (PAMPs) and catalyze the secretion of peptides into the hemolymph (Bechkage 2008, Tsakas and Marmaras 2010, Sompayrac 2012). This then activates (i.e. induces) transcriptional cascades that up-regulate the expression of antimicrobial peptides (AMPs) via immune response pathways that disrupt cell function or rupture the cell membrane (Irving et al. 2001, Bechkage 2008, Tsakas and Marmaras 2010). Genes that encode the synthesis of PRPs are unexpressed

(or constitutively expressed at low levels) prior to microbial exposure, at which point expression is induced (Dziarski and Gupta 2006). Two major groups of inducible PRPs associated with insect immunity are peptidoglycan-recognition proteins (PGRPs) and C-type lectins. PGRPs bind to specifically to peptidoglycans, which are components of bacterial cell membranes and mediate response to bacterial infections **(**Dziarski and Gupta 2006, Bechkage 2008). C-type lectins (i.e. immunelectins) are PRPs that bind to lipopolysaccharides and components of gram negative bacterial cell walls, activating the lectin-pathway via synthesis of lectin-pathway-specific serine proteases involved in coagulation and activation of the Toll pathway (Fujita 2002, Yu et al. 2002).

While innate immune response pathways are numerous and complex, complete with crosstalk between pathways (Tsakas and Marmaras 2010), transcription of genes encoding AMPs occurs mainly via two pathways: the Toll pathway and the Immune deficiency (Imd) pathway. The Toll pathway responds mostly to fungi and gram-positive bacteria associated antigens (e.g. peptidoglycans), and the Imd pathway responds to gram-negative bacteria (e.g. lipopolysaccharides) (Hoffmann and Reichart 2002, Tsakas and Marmaras 2010). Some examples of the large array of AMPs identified in insects are cecropin, attacin, and defensin in *Drosophila melanogaster* (Lemaitre and Hoffmann 2007), immunelectins and serine proteinases in Lepidopterans (Zhu et al. 2003) and gram-negative binding proteins (GNBPs), lectins, and ficolins in *Epirrita autumnata* (Rantala and Roff 2007).

A number of genes and peptides related to innate immunity have been described in termites. Among them are a transferrin gene that was up-regulated following exposure to an entomopathogenic fungus (Thompson et al. 2003, Rosengaus et al. 2007), termicin, spinigerin Lamberty et al. 2001) and GNBPs that can act as both PRPs and AMPs in several termite species

(Bulmer et al. 2009). Termites have been demonstratively immunized via exposure to sub-lethal dosages of pathogens, followed by the detection of inducible AMPs in the hemolymph (Rosengaus et al. 1998, Lamberty et al. 2001, Traniello et al. 2002, Rosengaus et al. 2007). In termites, production of AMPs via immune response pathways appears to differ significantly between not only species, but also between termites belonging to different colonies or separate castes (Rosengaus et al. 1999, Lamberty et al. 2001, Traniello et al. 2002). For example, Rosengaus et al. (2007) observed differential immune protein function among soldiers and pseudergates (i.e. false workers) with significant reduction of conidia viability in soldiers relative to that of immunized pseudergates.

1.3.2 Detoxification via Xenobiotic Metabolism

Similar to innate immunity, mechanisms of detoxification and xenobiotic (foreign chemical compounds) metabolism are widely conserved among invertebrates and vertebrates (Waxman 1992). Metabolism of xenobiotics and removal of metabolic by-products occurs via three primary phases (Feyereisen 2005, Oakeshott et al. 2005, Ranson and Hemingway 2005). Phase I enzymes catalyze the modification (i.e. oxidation, hydrolysis and/or reduction) of highly reactive compounds into less reactive, and less toxic, compounds (Zhu and Snodgrass 2003, Sun et al. 2006). They consist of ubiquitous, yet highly specialized, cytochrome P450 monooxygenases (CYP P450s), a widely abundant class of enzymes with a broad range of substrates (Zhu and Snodgrass 2003, Sun et al. 2006). Phase II enzymes are involved in conjugation (i.e. sulfation, acetylation and/or glucanization) and detoxification (i.e. hydrolysis) of the by-products from phase I (Sun et al. 2006, Willoughby et al. 2007). Glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) catalyze chemical reactions that add side groups to compounds and make them more hydrophilic (Haritos et al. 1994, Sun et al.

2006, Willoughby et al. 2007). Carboxylesterases are the phase II enzymes that catalyze hydrolysis, and subsequent detoxification, of esters (Willoughby et al. 2007). Point mutations in esterase genes have been shown to change their substrate specificity, resulting in esterase-based insecticide resistance (Hemingway 2000). Finally, it is in phase III that toxins are pumped out of cells and excreted via transmembrane proteins and malpighian tubules, respectively (Smith 1962).

Phenobarbital (PB), pentamethylbenzene (PMB), piperonyl butoxide (PBO) and atrazine are known as prototypical inducers for a broad variety of detoxification genes (Feyereisen 2005, Le Goff et al. 2006). A number of genes whose transcription is regulated by these inducers have been reported to be associated with insecticide resistance (Feyereisen 2005, Le Goff et al. 2006, Willoughby et al. 2007). For example, Willoughby et al. (2007) showed that exposure of *D. melanogaster* to PBO induced expression of 12 CYP P450 genes and five GST genes, a similar gene set to that induced by phenobarbital. DDT resistance in *D. melanogaster* is associated with induction and over-expression of genes involved in energy regulation; resistant flies had an increased metabolic rate relative to susceptible flies (Sun et al. 2006).

Detoxification gene families are rapidly evolving in insects, and there are few orthologs among species (Claudianos et al. 2006, Ranson et al. 2002). In FST, susceptibility to termiticides varies even among colonies (Gatti and Henderson 1996, Osbrink et al. 2001, Valles and Woodson 2002) as does enzyme activity (Valles and Woodson 2002). Differing levels of susceptibility have even been found among castes; Gatti and Henderson (1996) and Osbrink et al. (2001) suggest that workers are more resistant to insecticides than soldiers. Valles and Woodson (2002) observed that soldiers show only a fraction of detoxification enzyme activity relative to workers (Valles and Woodson 2002).

1.3.3 Functional Genomics

When studying genetic mechanisms, it is imperative to have a firm understanding of what is known as the central dogma of molecular biology. The building of proteins from genetic material involves several steps, including DNA replication, mRNA transcription, translation and processing (Harshman and James 1998). These steps can be further divided into two categories: gene regulation and gene expression. Transcription of mRNA is the point at which gene regulation occurs (generally in response to such factors as caste, life stage, physiology, tissue of origin, environmental signals, disease state or mutation (Harshman and James 1998, Pevsner 2003). The steps between gene regulation and gene expression are highly complex; examples of which include the up- or down-regulation of several coordinated genes and/or the activation of cascading biological pathways (e.g. the Toll and Imd pathways).

The emerging field of functional genomics provides tools needed to elucidate genetic underpinnings of immunology and detoxification. In contrast to structural genomics, which determines the sequence of genes, functional genomics is used to determine how genes function inside a living organism (Heckel 2003). In recent years, genomes of social insects have become available, e.g. *Apis mellifera* (The Honeybee Genome Sequencing Consortium 2006), and there are increasing numbers of cDNA and EST libraries being published for other social insects as well (e.g. *Bombus terrestris:* Pereboom et al. 2005 and *Vespula squamosal*: Hoffman and Goodisman 2007), including termites (e.g. Weil et al. 2007, Tartar et al. 2009, Steller et al. 2010). The most comprehensive genomic resource currently available for *C. formosanus* is the expressed sequence tag (EST) library that was developed by Husseneder et al. (2012) and contains several genes putatively involved in innate immunity (e.g. AMPs, lectins and serine proteases) and detoxification (e.g. CYP P450s and GSTs), among others. To date, functional

genomics in termites has been limited to investigations into caste regulation (Evans and Wheeler 2001, Miura et al. 2001, Koshikawa et al. 2005, Tarver et al. 2009, Ishikawa et al 2010, Tarver et al. 2012), digestion (Tartar et al. 2009) and reproduction (Weil et al. 2007, Korb et al. 2009, Husseneder et al. 2012). The aim of the current study was to employ functional genomics to elucidate differential expression of immune response and detoxification genes in workers and soldiers of *C. formosanus* colonies.

1.4 Research Goals and Objectives

The principal goals of this research were to identify genes associated with immunity and/or detoxification in FSTs, test whether expression can be induced by septic injury and/or xenobiotic challenge and to test the hypothesis that expression of immune response and/or xenobiotic metabolism genes is significantly increased in FST workers, relative to soldiers. Specific objectives are outlined below.

Objective 1: Identify genes associated with immunity and/ or detoxification in the FST and develop primers for these target genes. To achieve this objective, I screened the recently completed expressed sequence tag (EST) library of FST (Husseneder et al. 2012), primary literature and public databases for putative genes associated with immunity and detoxification. I then designed and tested primers for target genes for quantitative, real-time PCR (qRT-PCR).

Objective 2: Identify reference (aka "housekeeping") genes suitable for quantifying relative expression of target genes in FST workers and soldiers. To achieve this objective, I screened the recently completed expressed sequence tag (EST) library of FST (Husseneder et al. 2012), primary literature and public databases for housekeeping genes and designed primers for them. Putative reference genes were tested for efficiency, reproducibility, lack of primer-dimers, lack of non-specific binding and consistency across samples.

Objective 3: Design experiments to induce putative immunity and detoxification related target genes in FST workers and soldiers. To achieve this objective, I subjected FST workers and soldiers to sub-lethal doses of inducing agents via septic injury with non-pathogenic bacteria (*Escherichia coli* and *Pilibacter termitis*) or via no-choice feeding assays (serially-diluted phenobarbital).

Objective 4: Calculate relative expression of target genes and test for differences in expression of these genes between colonies, treatment groups and, ultimately, between castes. To achieve this objective, I isolated total RNA from FST workers and soldiers and used it to synthesize cDNA for qRT-PCR amplifications. The qRT-PCR amplification data for target and reference genes were used to calculate relative expression based on the methods described in Pfaffl (2001). Finally, relative expression values were compared via non-parametric, multivariate analysis of variance.

Chapter 2: Materials and Methods

2.1 Sample Collection

Workers and soldiers of the Formosan subterranean termite (FST) were collected from 3 established New Orleans Mosquito and Termite Control Board (NOMTCB) research sites in May 2009 (voucher specimens are deposited in the Louisiana State Arthropod Museum: LSAM). Figure 2.1 shows sample site locations which were as follows: (1) City Park, adjacent to Tad Gromley Stadium: 1 Palm Drive, (2) Louis Armstrong Park: 901 North Rampart Street and (3) The French Quarter: 300 Canal Street. Based on the evidence from previous investigations of FST colony size and flight distance (Husseneder et al. 2005, 2007, 2011, Messenger and Mullins 2005, Simms and Husseneder 2009), FSTs from these locations were genetically distinct from one another and were designated Colony 1, Colony 2 and Colony 3. Termites (~500 workers and 500 soldiers) were collected from an inground monitoring station (Sentricon, Dow AgroSciences LLC: Indianapolis, IN) at each sample site and then transferred to plastic tubs (50×30×20cm) lined with moist corrugated cardboard and maintained overnight in the laboratory at 25°C and 30% relative humidity (Intellus Environmental Controller, Percival: Perry, IA) .

2.2 Mortality Assays to Determine Sublethal Treatment Conditions

2.2.1 *Escherichia coli* and *Pilibacter termitis* Treatments

A pilot study was conducted to modify published methods (Calleri et al. 2006) to determine the appropriate sublethal treatment for inducing immunity-related gene expression. Non-pathogenic, gram-negative *Escherichia coli* and non-pathogenic, gram-positive *Pilibacter termitis* (ATCC BAA-1030) were used to experimentally infect termites. These bacteria naturally occur in the termite gut and were cultured from FST in previous studies (*E. coli*: Husseneder

Figure 2.1: Sample Collection. Aerial view (*Source:* "New Orleans, LA." 29˚58'35.33"N, 90˚03'33.51"W. Google Earth. November 29, 2011. June 14, 2012) of NOMTCB research sites from which FST workers and soldiers were collected in May 2009. The 3 inground stations are denoted with circles and numbered (1, 2 and 3) for colony designation.

et al. 2009, *P. termitis*: Higashiguchi et al. 2006). *E. coli* was cultured in the laboratory under aerobic conditions in 1 mL of BHI broth for 1 day on a shaking incubator at 30˚C. *P. termitis* was grown anaerobically, using H_2/CO_2 BBL GasPaks™ (Becton Dickinson and Company: Franklin Lakes, NJ), in 1 mL of BHI broth at 30˚C for 3 days. Sterile 28 gauge needles (Comfort Point: Nanaimo, British Columbia) were each soaked for approximately 60 seconds with equal volumes of exponential growth phase *E. coli* and *P. termitis* (200 µL supernatant of each, placed in a 1.5 mL micro-centrifuge tube). Termites (sampled from Colony 1: Fig 2.1) were cold immobilized on a thermoelectric laboratory chill table (Bioquip: Gardenia, CA) and placed with their ventral surface exposed on a dissecting microscope stage (Leica MZ7.5 Stereomicroscope, Meyer Instruments: Houston, Texas). The abdomen was swabbed with 70% ethanol before being pierced through the intersegmental membrane of the 4^{th} and 5^{th} segments with the septic needles (Fig 2.3). This was done for 20 workers and 20 soldiers in total. Following inoculation with bacteria, termites were isolated in 47 mm Petri dishes and kept at 25°C and 30% relative humidity (Intellus Environmental Controller, Percival: Perry, IA) for 48 hours. Twenty workers and 20 soldiers that were not inoculated with bacteria (controls) were held in separate dishes under the same conditions. Mortality was determined as the inability for the termite to right itself after 30 seconds and was assessed for treated and untreated termites at 12, 24 and 48 hrs. It was determined that the 24 hr. treatment would be used as the sublethal treatment condition for target gene induction (see Table 3.1), which is consistent with the rapid induction time of insect immune systems (i.e., peptide synthesis in *D. melanogaster:* Fehlbaum et al. 1994, and in *P. spiniger:* Lamberty et al. 2001).

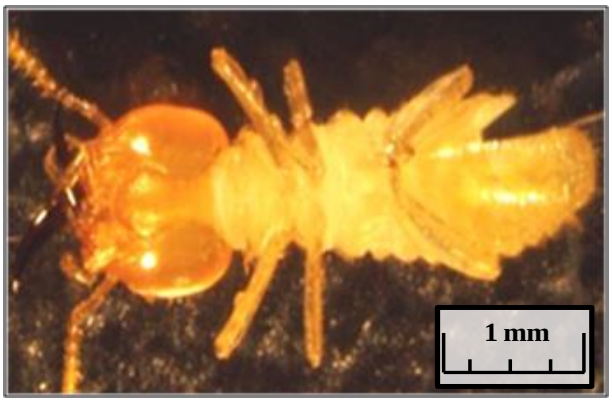

Figure 2.2: Injection of *E. coli/ P. termitis*. A cold immobilized FST soldier is inoculated with *E. coli* and *P. termitis* via septic needle, under a dissecting microscope, 10 X magnification.

2.2.2 Phenobarbital Treatments

To determine the sublethal dose and time for induction of target genes via experimental treatment with PB (Sigma-Aldrich: St. Louis, MO), a simple and efficient method of feeding PB to the termites was first established as follows: 1) Termites from Colony 1 (Fig 2.1) were each placed in an individual 47 mm Petri dish (Millipore: Billerica, MA, Fig 2.2),
2) PB was diluted to a 1% concentration with distilled water, 3) PB-infused water was pipetted directly onto the inside surface of each Petri dish in 2 µL droplets, 5 droplets per dish, 4) 1 % PB was also added, in 2 µL droplets, to Petri dishes that did not contain a termite and 5) All of the dishes were kept at 25°C and 30% relative humidity (Intellus Environmental Controller, Percival: Perry, IA) for 48 hrs. In total, there were 60 Petri dishes in the experiment; 20 each contained a worker, 20 each contained a soldier, and 20 control dishes each contained no termite. The PB-infused water droplets were monitored at 12 hr. intervals. Visual comparisons were made between controls and dishes containing a termite worker or soldier that showed that the level of evaporation was negligible over the 48 hr. incubation period, and the 1% PB droplets were consumed at the same rate by termites of each caste.

The next step was to determine the maximum concentration and time at which termites were undergoing deleterious effects of PB exposure but were not yet dying off. This sublethal treatment would be ideal for investigating induced expression of target genes that are likely associated with detoxification in termites. Termites from Colony 1 (Fig 2.1) were individually placed in 47 mm Petri dishes (Millipore: Billerica, MA, Fig 2.2), kept at 25°C and 30% relative humidity (Intellus Environmental Controller, Percival: Perry, IA) for 48 hrs. PB was serially diluted with distilled water (1%, 0.5%, 0.25% and 0.125%), and each concentration of PB was fed to termites [(20 workers + 20 soldiers) * (4 dilutions of PB) = 160 termites in total] in 2 µl droplets. Controls were fed distilled water only (20 workers + 20 soldiers = 40 termites in total). Mortality was determined as the inability for the termite to right itself after 30 seconds and was assessed for treated and untreated (control) workers and soldiers at 12, 24 and 48 hrs. Based on the results of this experiment (see Table 3.1), the 0.5% PB treatment at 24 hrs. was chosen as the sublethal treatment condition for target gene induction in both workers and in soldiers, which lies within the published time frame recommended for measuring PB- induced gene expression in insects: from 4 hrs. in *D. melanogaster* (Willoughby et al. 2007) to 5 days in cockroaches (Brown et al. 2003).

2.3 Experimental Design for Measuring Gene Expression

Once the appropriate sublethal treatment conditions were established (see section 2.2), experimental conditions were designed to test: 1) controls vs. 0.5% PB treated termites, 2) controls vs. *E. coli/ P. termitis* treated termites: 3) 0.5% PB treated termites vs. *E. coli/ P. termitis* treated termites 4) 0.5% PB treated workers vs. 0.5% PB treated soldiers and 5) *E. coli/ P. termitis* treated workers vs. *E. coli/ P. termitis* treated soldiers. The objectives were to first determine constitutive (transcribed continually) expression of target genes and compare that to

induced (transcribed upon activation) expression of target genes. Next, induced expression was compared between treatments (0.5% PB and *E. coli/ P. termitis*) and between castes (workers and soldiers).

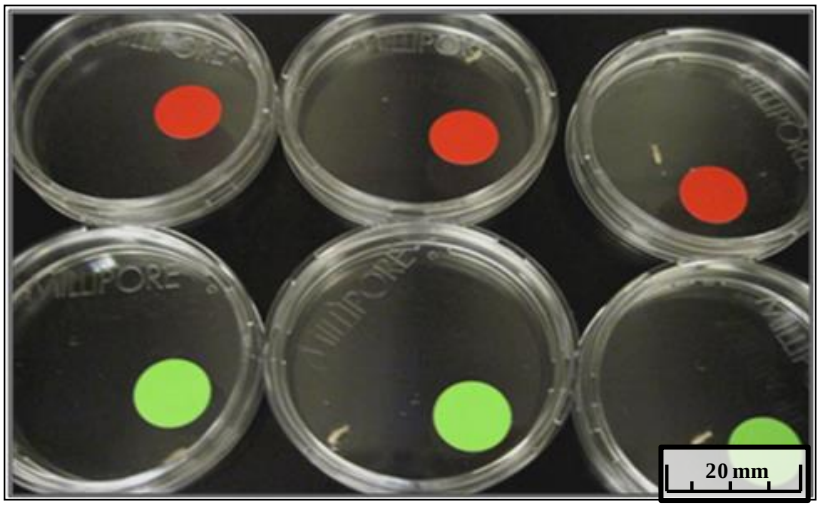

Figure 2.3: No-choice PB Feeding Assays. FST workers and soldiers were isolated in Petri dishes. Treated termites were fed 2ul droplets with known concentrations of PB (red), and untreated controls were fed distilled water (green). Incubation was 48 hrs., 25°C and 30% RH.

Twenty workers and 20 soldiers per colony (Colony 1, Colony 2 and Colony 3: Fig 2.1) were fed 0.5% PB, 20 workers and 20 soldiers per colony were inoculated with the *E. coli/ P. termitis* mixture and 80 termites [(20 workers + 20 soldiers) * (2 treatments) = 80 total termites] were left untreated (controls). Termites were each placed in individual 47 mm Petri dishes (Millipore: Billerica, MA) and kept at 25°C and 30% relative humidity (Intellus Environmental Controller, Percival: Perry, IA) for 24 hrs. All termites were then flash-frozen at -80˚C (Ultra low VIP, Sanyo Scientific: Wood Dale, IL). For each of the 3 colonies (3 biological replicates), five 0.5% PB treated workers, 5 *E. coli/ P. termitis* treated soldiers, 10 control workers and 10 control soldiers (5 of each caste per treatment type) were randomly chosen as technical replicates for qRT-PCR reactions.

2.4 RNA Isolation, cDNA Synthesis and Thermal Conditions

Total RNA was extracted from whole bodies using the RNA Purification Microkit (QIAGEN, Valencia, CA). Individual termites were homogenized in 150 μL of lysis buffer and stored on ice. Next, 295 μL of RNAse free water and 5 μL of Proteinase K were added. Samples were vortexed for 5 sec, incubated in a 55˚C water bath for 10 min and centrifuged at 6000 rpm for 3 min. Supernatants were transferred to new tubes and 100% EtOH was added in increments of 0.5 μL per 1 μL of supernatant. Samples were pipette-mixed, transferred to spin columns, spun for 15 sec, and the flow through was discarded. Then, 350 μL of washing buffer was added, the samples were spun for another 15 sec, and the flow through was again discarded. Ten microliters of DNase and 70 μL of binding buffer were then added, and samples were incubated at room temperature for 15 min. The spin columns were washed once again with 350 μL of washing buffer before being transferred to new collection tubes and being washed with 500 μL of elution buffer. Finally, the spin columns were washed with 500 μL of 80% EtOH, spun for 2 min, and the flow through was discarded. Samples were eluted by adding 14 μL of RNase free water directly to the filter of each spin column and then centrifuging the samples at maximum speed for 1 min.

RNA concentrations were assessed at the 260/280 nm ratio of absorbance using a ND-1000 spectrophotometer (Nanodrop Technologies: Wilmington, DE). RNA samples were normalized to 10 ng per μl by adding the appropriate amount of RNAse free water and were then used as template for complimentary DNA (cDNA) synthesis via the iScript Kit (Biorad: Hercules, CA, USA). Reverse transcription PCR (RT-PCR) reactions each consisted of 10 μL of normalized RNA template, 3 μL of RNAse free water, 4 μL of 5X iScript Rxn Mix, 2 μL of

Oligo d(T) primer and 1 μL of iScript Reverse Transcriptase for a total volume of 20 μL. The RT-PCR program (42˚C for 90 min followed by 85˚C for 5 min) was run on a PTC-200 DNA Engine gradient thermocycler (MJ Research: San Francisco, California).

The cDNA was normalized to 1,000 ng per μl and used as template for qRT-PCR. Amplifications were performed using the IQ5 iCycler (Biorad: Hercules, CA, USA) in reactions each containing 5 μl cDNA, 25 μl SYBR-green Supermix (BioRad), 150 nm forward and reverse primer and 17 μl H_2O for a total volume of 50 μl. The thermal protocol consisted of one cycle of 95˚C for 3 min. followed by 40 cycles of 1) 95˚C for 10 sec and 2) 58˚C for 30 sec. RFUs (relative fluorescence units) and Ct values (fractional cycle at which amplification reaches a detection threshold) were recorded at the end of each cycle by the iCycler software (Fig 2.4).

2.5 Candidate Genes and Primer Design

2.5.1 Target Genes

There were a total of 10 candidate target genes used in this study. Eight genes (Accession numbers: FK835449, FK833823, FK833694, FK835436, FK832766, FK834461, FK835521 and FK836944) were selected from the EST library for FST (Husseneder et al. 2012) that were most likely to be induced via detoxification mechanisms and/ or immune response (based on annotation of the EST library and mining the literature and public databases for similar genes). Candidate genes (Table 2.1) included those from 2 Cytochrome P450 families (CYP 15A1 and CYP 314A1), a multi antimicrobial extrusion protein gene (MatE 1), a gene expressing a cell adhesion molecule (DSCAM Ig7A), 2 lectin-like genes for binding lipopolysaccharides (LBP and Lectin C), a ficolin precursor (Ficolin 2) and a gene that expresses a chitin-binding protein Peritrophin-A (CBP). Primer pairs (3 per gene) were designed (PRIMER-BLAST

software suite, NCBI) to have between 17 and 28 bases, with ~50% to 60% GC content, along with two additional primers (glutathione s- transferase: GST and gram- negative binding protein 2: GNBP 2) developed for ESTs from other termite species (GST: *Hodotermopsis sjoestedti* and GNBP: *Drepanotermes rubriceps*). These two primers were previously developed and successfully used to examine differential gene expression among FST queens and precopulatory females and to test for differential gene expression between lab reared and field collected FST workers and soldiers (Husseneder et al. 2012).

Each primer was tested using qRT-PCR (see Section 2.4 for extraction procedures and thermal conditions) with cDNA from three untreated, randomly chosen workers from Colony 1 (Fig 2.1) as template. For each gene, one of the three primer pairs was selected based on the following criteria (Bustin et al. 2009, Ramakers et al. 2003, Ruijter et al. 2009): 1) consistency across technical replicates, 2) target amplicon at 50 to 100 bp, 3) melting curve with a single peak and 4) reaction efficiency (E) $\geq$1.8. Table 2.1 lists each candidate target gene and the corresponding primer sequences that passed all of the above criteria (see Appendix B for a comprehensive list of designed and tested primers).

2.5.2 Reference Genes

Reference genes used to quantify target gene expression must be reliable, highly specific and minimally influenced by experimental conditions (Bustin et al. 2009, Radonic et al. 2004, Silver 2006). Although there are many reference genes that have been used to quantify differential expression, these genes are not universal. Furthermore, it has been proposed that not only should candidate reference genes be subjected to identical experimental conditions as target genes (Bustin et al. 2009, Infante et al. 2008, Radonic et al. 2004), but also that two or more

types of reference genes should be used in qRT-PCR studies (Bustin et al. 2009, Infante et al. 2008, Thellin et al. 1999, Vandesompele et al. 2002). Therefore, primer pairs designed for putative reference genes were rigorously tested for efficiency and reliability.

The EST library for FST (Husseneder et al. 2012) was first screened for reference genes used in previous studies for quantifying gene expression, i.e. glyceraldehyde-3-phosphate dehydrogenase (FK834351: Dheda et al. 2004, Infante et al. 2008, Radonic et al. 2004, Silver et al. 2006, Vandesompele et al. 2002), TATA-binding protein (FK835420: Dheda et al. 2004, Vandesompele 2002), Elongation factor-1-alpha (FK834645: Dedha et al. 2004, Guenin et al. 2009, Infante et al. 2008, Nicot et al. 2005) and Ubiquitin specific peptidase (FK831079: Guenin et al. 2009, Infante et al. 2008, Nicot et al. 2005).

Next, the library was screened for genes most likely to be involved in cell maintenance and/ or basal cell metabolism, i.e. "housekeeping" genes. Cytoplasmic heat shock protein and NADH dehydrogenase (Accession numbers: FK835495 and FK833785) were selected, bringing the total number of putative reference genes to 6). Fifteen primer pairs (2-3 per reference gene) were designed, using PRIMER-BLAST (NCBI), to have between 17 and 28 bases, 50% to 60% GC content and target amplicons of 50 to 100 bp. Primer testing was performed using qRT-PCR (see Section 2.4 for extraction procedures and thermal conditions) with cDNA template synthesized from Colony 1 workers and soldiers (Fig 2.1); 3 termites from each caste were each randomly chosen from the 0.5% PB treatment group, the *E. coli/ P. termitis* treatment group and the untreated control group. Primers for reference genes needed to pass all of the same criteria given for target gene primers (see Section 2.5.1) while also being consistent across treatment types (0.5% PB and *E. coli/ P. termitis*), among castes (workers and soldiers) and across plates.

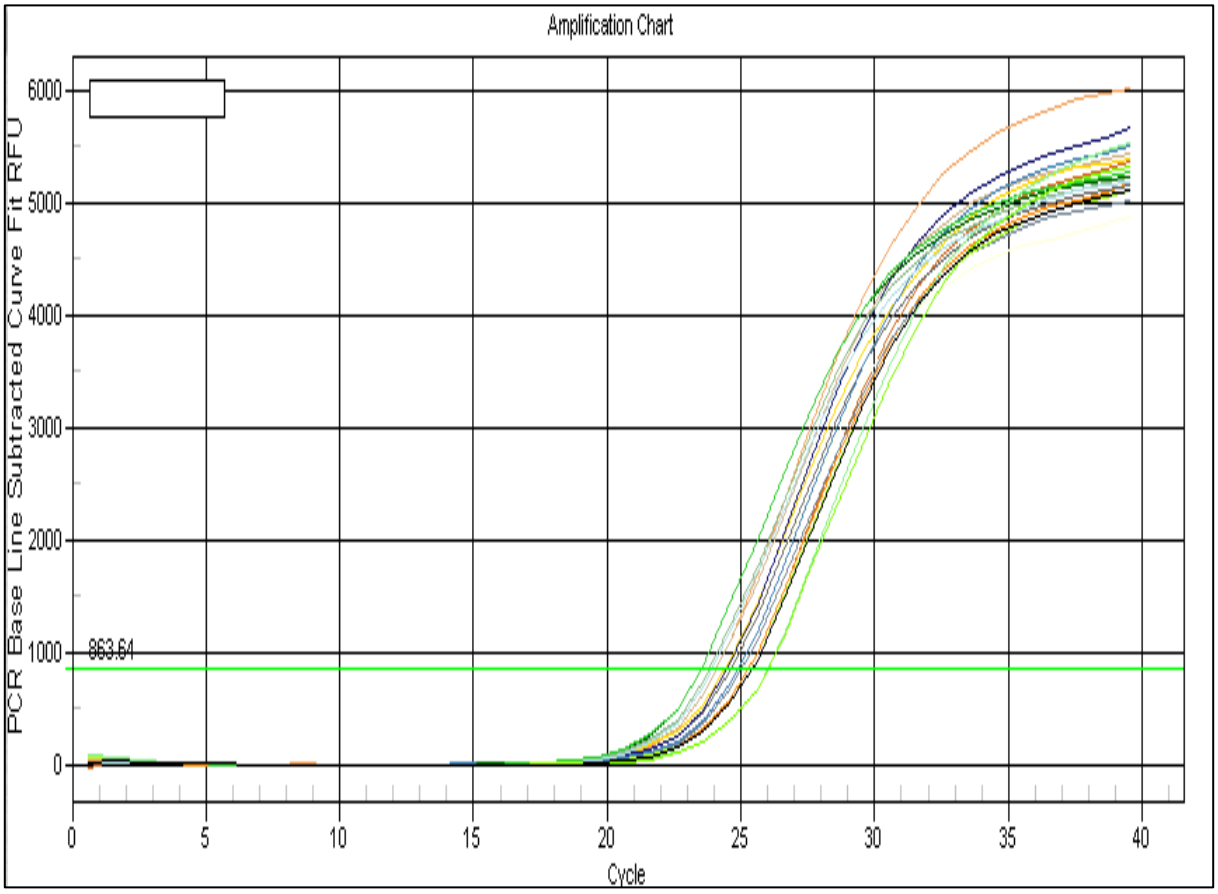

Figure 2.4: Screenshot of iCycler Data. **R**eference gene primers were tested for consistency across colonies, castes and treatments (3x2x2= 12 reactions using primers designed for the cytoplasmic heat shock protein are shown).

Appendix B lists each of primer pairs designed and tested for the reference genes, along with their melting points, sequences and GC content. Of the 15 total primer pairs that were tested, only 3 passed all of the criteria listed above (cytoplasmic heat shock protein, NADH dehydrogenase subunit 4 and elongation factor 1-alpha: Table 2.1). These primer sequences were shown to amplify at high efficiency and stability across not only technical replicates, but also across biological replicates, making them well suited for use in this study (see Fig. 2.4 above).

2.6 Relative Expression and Tests for Differential Expression

Relative expression (R) was calculated using the most conservative methods available, according to the current literature. For instance, to account for the slight variations among qRT-PCR amplifications, LinRegPCR software (Ramakers et al. 2003, Fig 2.5) was used to calculate the individual efficiencies (E) for reactions (Fig 2.2), which were then averaged across the 5 technical replicates for each gene and treatment type (see Table 5.2). C_T values (cycle at which the amplification curve reaches the detection threshold) given by the iCycler qRT-PCR software (Fig 2.1) were similarly averaged across technical replicates (Table 5.2). For the 3 reference genes, geometric means (normalization factor: NF_n, n= 3, Vandesompele et al. 2002) of C_T and E values were calculated (Table 3.3), and these geometric means were next used to calculate relative expression (R) of target genes.

R values were calculated based on the methods described in Pfaffl (2001), in which E values for target and reference samples are not assumed to be equal [$(R) = E_{target}^{C_T,target\ (control-sample)}/E_{ref}^{C_T,ref\ (control-sample)}$] where *target* refers to the target gene under investigation (Table 2.1) and *ref* denotes the geometric mean of C_T and E values for the 3 reference genes (run under the same experimental and reaction conditions as the target amplicons). When calculating R values for constitutive expression, *control* refers to a non-template control (i.e. no cDNA added to the qRT-PCR reaction), and *sample* refers to a biological control (i.e. untreated termites). However, when calculating R values for induced target gene expression, *control* refers to untreated termites, and *sample* refers to a treatment type (i.e. termites treated with 0.5% PB or *E. coli/ P. termitis*). R values were compared between constitutively expressed and induced target amplicons within and between 1) colonies, 2) treatment groups and 3) castes via non-parametric, multivariate analysis of variance (PROC GLM, SAS/STAT Software 9.3, SAS Institute Inc.,

2001). Furthermore, the GLIMMIX and RANK procedures were used to analyze non-normally distributed data points. The statistical model used was: gene = 1 to 10; expressed(gene) = tmt cas tmt*cas; random tmt*cas*col, where "gene" refers to the 10 target genes, "tmt" refers to treatment group (untreated control, 0.5% PB or *E. coli/ P. termitis*), "cas" is caste (worker or soldier), "col" refers to colony (Colony 1, 2 or 3) and "expressed(gene)" is the interaction of treatment, caste and colony. Differences in expression were considered significant when $P < 0.05$.

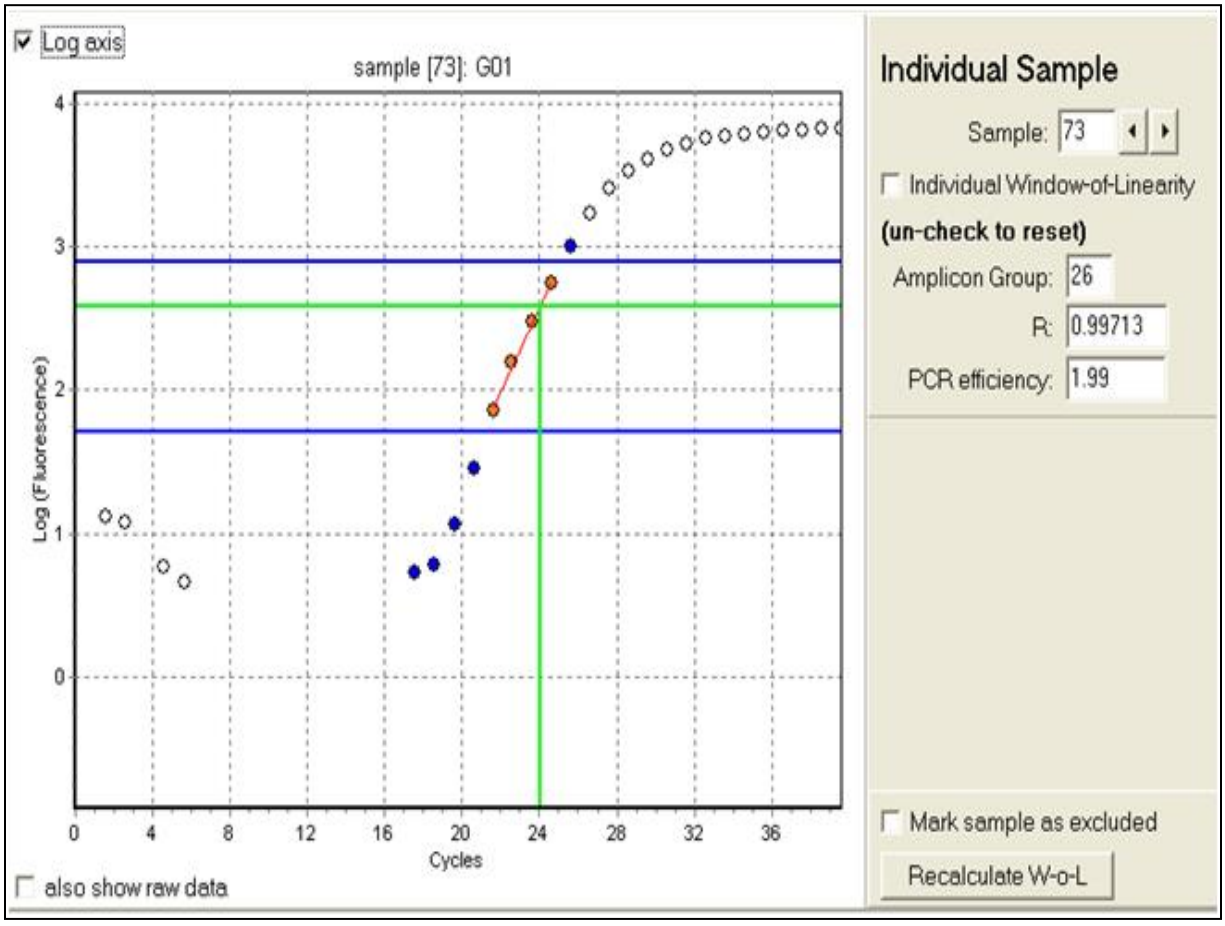

Figure 2.5: Screenshot of LinReg PCR Data. Shown is the individual window-of-linearity (w-o-L) and individual reaction efficiency (E= 1.99) for an amplification curve given by the iCycler qRT-PCR program (BioRad).

Table 2.1: Primer Descriptions for Putative Target and Reference Genes. Listed are genes putatively associated with FST detoxification (top) and immunity (middle). Primers were selected based on testing criteria outlined in section 2.5.1. Primers for selected reference genes (bottom) were chosen based on testing criteria outlined in section 2.5.2.

GENE	Closest Match: UniProt	Species	FUNCTION	ACCESSION NO.	PRIMER SEQUENCE (5'-3')	Tm
CYP 15A1	Cytochrome P450 15A1	*Reticulitermes flavipes*	Cytochrome P450, epoxidase, JH synthesis, caste regulation	FK835449	ATTTGTCACTCGCCTTGGTC AAAGTTGCCATACGTGGAGG	60.1 60.0
CYP 314A1	Cytochrome P450 314A1v2	*Laodelphax striatella*	Cytochrome P450, oxidoreductase, ecdysone 20-monooxygenase linkage	FK833823	ACTGGTTTGTAGTCGGTGCC TTAGCACCGAGAGACGTTCA	60.0 59.6
MatE 1	GF24314	*Drosophila ananassae*	Cationic efflux transport pump, sodium antiporter, excretion of organic cations	FK833694	TTGACACAACACCTTTCGGA GCCGTGTTTGAGGATGAAAT	60.1 59.9
GST	Glutathione s-transferase class theta	*Blatella germanica*	Glutathione s- transferase, metabolises lipophilic toxins, excretes xenobiotics	DC239424	GTTTCAAGCCTCGCTTTGAC CGTCACTGTGAGACTGCCAT	60.0 59.9
DSCAM Ig7A	AbsCAM Ig7A	*Harpegnathos saltator*	Down syndrome cell adhesion molecule axon targeting, Fibronectin type 3	FK835436	CCGTACCATTGACGGAGAGT CACTTGTGTTGCCCGTAATG	60.0 60.0
GNBP 2	GNBP 2	*Coptotermes formosanus*	Gram negative binding protein, immune signalling, pattern recognition receptor	DQ058934	CTTCCCAGTGTCGAAAGCTC TGGGTACCCCATAGGAGATG	60.0 59.6
CBP	Peritrophic matrix protein 9	*Tribolium castaneum*	Chitin binding protein, mucin-like matrix protein, Peritrophin-A	FK832766	CGGGAGGACTGCTAGAACTG TGAGGGAACTCCCTGTTCAC	60.0 60.1
LBP	Hemolymph LPS-BP	*Blatta americana*	Lipopolysaccharide binding protein Lectin-like, hemolymph	FK834461	CTTCCCAGTGTCGAAAGCTC TGGGTACCCCATAGGAGATG	55.7 55.5
Lectin C	C-type lectin 11	*Bombyx mori*	Galactose binding, pattern recognition receptor	FK835521	AGGGTGAAAGAGGAGCAACA TAAAAATCCCAACAACCCCA	59.8 60.0
Ficolin 2	Ficolin 2	*Pediculus humanus*	Antigen recognition, lectin pathway complement	FK836944	CTCCGTCGGACTCTCATCTC GCGCTATAACCTGGGTCTCA	56.4 56.6
HSP	70kD heat shock protein	*Scylla paramamosain*	ATP and nucleotide binding	FK835495	AGCCTTGGCCACAACAGTGCAA GGAGCAGGAGCAGGACCGACT	59.7 59.9
NADH	NADH dehydrogenase subunit 4	*Coptotermes formosanus*	Mitochondrial electron, sodium ion and proton transport, ubiquinone activity	FK833785	ACGAAGCAACCCATAACCACCAAGC GGGCTCATGTTGAGGCTCCTGTT	59.9 58.9
EFA	Elongation factor 1-alpha	*Diaphorina citri*	GTP binding, regulation of tranlational elongation	FK834645	GGCGGTCACATTTCTCCTTA CTGAACCACCCTGGTCAGAT	54.8 56.6

Chapter 3: Results

3.1 Mortality Assays and Sublethal Treatments

The results of mortality assays to determine the appropriate sublethal treatment conditions are given in Table 3.1, which lists percent mortalities for each treatment and observation time. The highest observed mortality for the PB treatments was 100% for workers and soldiers fed with 1% PB and held for 48 hrs. The highest observed mortality for the *E. coli/ P. termitis* treatments was 70% for soldiers at 48 hrs. One hundred percent of control workers and soldiers survived over the 48 hour period.

3.2 Amplification of Target and Reference Gene Transcripts

A total of 10 genes putatively involved in immunity and/or detoxification in termites were examined in this study (see Table 2.1). Mean C_T values (Table 3.2) for target amplicons ranged from 25.89 to 37.32 (mean= 31.28 $\pm$ 5.83). Individual reaction efficiencies (E: Table 3.2), ranged from 1.69 to 2.19 (mean= 1.96 $\pm$ 0.07). The only qRT-PCR reactions that did not amplify target gene transcripts were those that did not contain cDNA template, i.e. non-template controls (NTC: Fig 3.1), in which case C_T values were reported as zero or, alternatively, unusually high (> 30 cycles), and E values were unusually low (1.6 or below).

Amplifications of reference gene transcripts were consistent across treatment types (0.5% PB and *E. coli/ P. termitis*), among castes (workers and soldiers) and across plates (Table 3.3). Reference amplicons also showed high efficiency and stability across technical and biological replicates. Geometric means (normalization factor: NF_n, n= 3) of C_T and E values for reference gene transcripts were calculated according to treatment type (Table 3.3).

Table 3.1: Mortality of FST Workers and Soldiers**.** Termites were fed serially diluted PB, injected with *E. coli* and *P. termitis* or were untreated (Control). Highlighted are sublethal treatments and control conditions for target gene induction.

Time (hrs)	Treatment Type	Mortality (%) Workers	Soldiers
48	1% PB	100	100
24	1% PB	70	70
12	1% PB	50	70
48	0.5% PB	50	50
24	0.5% PB	0	0
12	0.5% PB	0	0
48	0.25% PB	0	0
24	0.25% PB	0	0
12	0.25% PB	0	0
48	0.125% PB	0	0
24	0.125% PB	0	0
12	0.125% PB	0	0
48	*E. coli/ P. termitis*	50	70
24	*E. coli/ P. termitis*	0	0
12	*E. coli/ P. termitis*	0	0
48	Control	0	0
24	Control	0	0
12	Control	0	0

3.3 Relative Expression and Differential Expression

R values were compared between constitutively expressed and induced target amplicons within and between 1) colonies, 2) treatment groups and 3) castes via non-parametric, multivariate analyses of variance (SAS/STAT Software 9.3, SAS Institute Inc., 2001). Constitutive (untreated controls) expression of target gene transcripts was low, overall, (mean R= 0.014 $\pm$ 0.163) as compared to R values for reference genes (Table 3.4). All but 2 target genes were inducible, either with 0.5% PB or *E. coli/ P. termitis* (Table 3.4). The genes that were not induced by either 0.5% or *E. coli/ P. termitis* treatment were CYP 314A1 and DSCAM Ig7A.

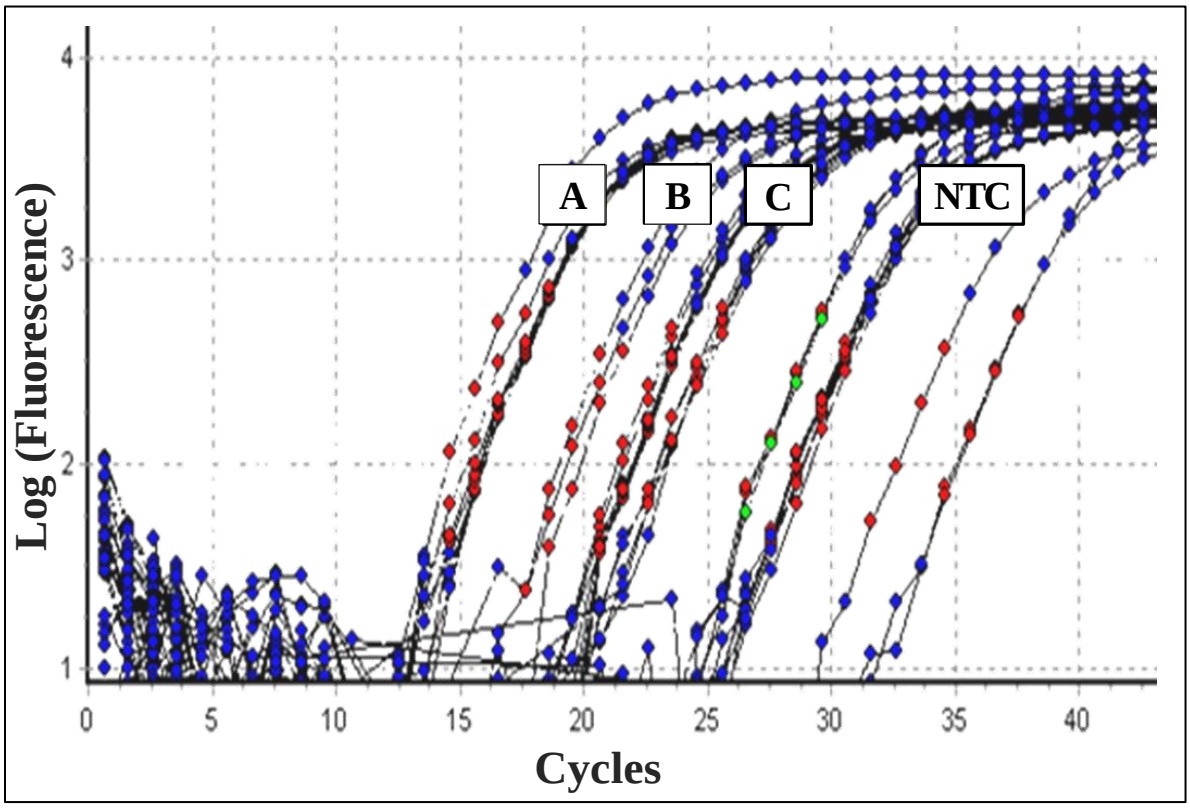

Figure 3.1: Baseline corrected (LinReg PCR) qRT-PCR amplification curves. Shown are curves for reference gene amplicons (A, B and C = NADH dehydrogenase subunit 4, Cytoplasmic heat shock protein and Elongation factor 1-alpha, respectively) and non-template controls (NTC). Data points inside the w-o-L are in red. These data were used to calculate E values for individual reactions. Data points in green highlight a reaction curve selected at the time this screenshot was taken.

Genes induced with the 0.5% PB treatment were CYP 15A1, GST, GNBP 2, CBP, LBP, Lectin C and Ficolin 2. Genes induced with the *E. coli/ P. termitis* treatments were CYP 15A1, MatE 1, GNBP 2, LBP, Lectin C and Ficolin 2. Table 3.4 gives the R values for target expression for termites treated with either 0.5% PB (mean= 2.014 $\pm$ 1.379) or *E. coli/ P. termitis* (mean R= 2.668 $\pm$ 1.988).

Table 3.2: Mean C_T and E Values for Target Gene Amplicons. C_T and E values were averaged across 5 technical replicates and are listed for each target gene transcript, treatment type, caste and colony. W1, W2, W3 = workers, and S1, S2, S3 = soldiers from Colony 1, 2 and 3, respectively.

GENE	TREATMENT	W1		W2		W3		S1		S2		S3	
		C_T	E	C_T	E	C_T	E	C_T	E	C_T	E	C_T	E
CYP 15A1	0.5% PB	25.33	2.04	25.03	1.99	25.82	2.06	23.84	2.05	26.06	2.06	24.00	2.04
	Control	34.52	1.98	34.71	1.97	36.80	2.05	35.74	1.97	33.87	1.96	41.82	1.96
CYP 314A1	0.5% PB	24.85	2.00	23.84	2.08	23.15	2.04	23.27	2.08	24.76	2.02	22.50	1.99
	Control	27.79	1.93	27.45	1.90	24.12	2.01	26.76	1.97	27.23	1.99	23.00	1.88
MatE 1	0.5% PB	35.56	2.03	31.95	1.99	36.61	1.97	31.37	2.01	34.05	1.98	32.40	2.05
	Control	36.49	1.97	32.08	2.03	42.64	2.00	31.91	2.09	32.80	2.01	37.89	2.10
GST	0.5% PB	30.16	1.81	28.84	1.97	33.93	1.91	29.53	2.16	44.47	1.71	33.52	1.99
	Control	40.54	1.85	38.86	1.84	36.18	1.76	29.36	2.22	45.48	1.70	33.36	2.03
DSCAM Ig7A	0.5% PB	26.34	2.03	29.74	1.91	27.09	2.02	24.87	1.93	26.76	2.01	24.31	2.02
	Control	31.80	1.91	30.70	1.91	31.54	1.97	30.39	1.94	31.15	1.99	31.39	1.95
GNBP 2	0.5% PB	25.04	2.01	24.57	2.00	26.95	1.96	32.55	1.90	32.04	1.95	32.26	1.93
	Control	34.19	1.92	33.71	1.95	36.82	1.83	34.31	1.97	34.44	1.97	41.93	1.95
CBP	0.5% PB	24.78	2.01	24.91	1.93	26.75	2.02	23.87	2.02	27.57	1.92	25.94	1.96
	Control	36.85	1.98	35.73	2.08	35.73	2.08	35.94	1.96	34.98	1.96	36.06	1.93
LBP	0.5% PB	23.12	1.97	25.86	1.97	24.31	2.01	21.06	2.06	22.07	1.99	22.20	2.01
	Control	41.99	1.90	41.99	1.88	31.51	1.94	32.46	1.97	32.06	1.95	34.53	1.96
Lectin C	0.5% PB	27.02	2.06	27.99	1.88	26.89	2.05	24.76	2.09	21.88	2.03	24.62	1.98
	Control	30.17	1.99	28.75	1.96	38.43	2.03	35.88	1.97	36.16	1.93	34.73	1.98
Ficolin 2	0.5% PB	26.42	2.04	22.88	1.90	26.05	2.00	23.81	2.00	25.84	2.04	25.18	1.96
	Control	30.67	1.98	29.78	1.97	36.57	1.92	35.04	2.03	35.10	1.97	34.53	1.95
CYP 15A1	*E. coli/ P. termitis*	25.25	1.99	24.53	2.02	23.34	1.97	24.19	2.00	24.50	2.00	28.75	1.80
	Control	38.81	1.69	38.56	1.89	36.19	1.93	35.06	1.94	36.34	1.87	37.17	1.87
CYP 314A1	*E. coli/ P. termitis*	25.60	1.93	25.53	2.04	24.31	1.99	23.93	1.97	23.27	2.08	23.13	2.07
	Control	31.57	1.96	27.91	2.03	29.63	2.00	31.56	1.97	28.86	1.98	27.26	2.06
MatE 1	*E. coli/ P. termitis*	22.66	1.95	23.89	1.90	34.91	1.97	34.83	1.97	33.78	1.96	33.01	1.99
	Control	38.15	1.71	38.01	1.84	43.58	2.01	38.68	2.01	38.74	2.08	40.09	1.99
GST	*E. coli/ P. termitis*	40.39	1.91	40.02	1.86	35.05	1.93	40.08	1.84	38.47	1.90	39.05	1.91
	Control	41.30	1.84	41.22	1.93	37.91	1.84	40.77	1.71	40.01	1.94	37.11	2.04
DSCAM Ig7A	*E. coli/ P. termitis*	27.06	1.99	24.45	1.96	26.14	1.94	26.61	1.91	25.01	1.92	25.61	2.00
	Control	35.37	1.88	32.68	1.95	33.03	1.85	32.93	2.06	32.34	1.95	32.95	1.93
GNBP 2	*E. coli/ P. termitis*	27.34	1.99	26.61	1.96	36.44	1.93	32.96	1.95	31.87	2.01	32.36	1.89
	Control	40.21	1.93	41.73	1.97	41.56	1.92	32.14	2.04	32.14	2.04	35.16	1.94
CBP	*E. coli/ P. termitis*	26.64	1.73	28.16	1.99	24.40	1.91	26.94	1.98	25.84	1.99	23.44	1.94
	Control	36.03	1.99	36.03	1.89	39.14	1.96	38.15	1.71	38.62	1.97	41.64	1.94
LBP	*E. coli/ P. termitis*	23.92	1.99	24.87	1.98	26.00	1.94	26.07	1.99	24.92	2.05	28.11	1.91
	Control	35.66	1.82	36.81	1.88	33.60	1.93	36.92	1.87	36.92	1.99	35.17	1.98
Lectin C	*E. coli/ P. termitis*	25.71	2.07	25.46	2.01	25.32	1.96	24.28	2.05	25.75	2.07	26.23	2.04
	Control	36.62	1.87	35.90	2.03	35.90	2.03	36.35	1.86	36.29	1.93	37.50	2.05
Ficolin 2	*E. coli/ P. termitis*	26.23	2.01	27.27	1.89	24.45	2.01	24.24	2.04	26.25	2.02	26.71	2.00
	Control	35.78	1.93	35.70	1.95	35.75	1.98	31.79	1.91	32.08	2.03	31.84	2.00

Table 3.3: Mean C_T and E Values for Reference Gene Amplicons. C_T and E values were averaged across 5 technical replicates and are listed for each reference gene transcript, treatment type, caste and colony. W1, W2, W3 = workers, and S1, S2, S3 = soldiers from Colony 1, 2 and 3, respectively.

GENE	TREATMENT	W1		W2		W3		S1		S2		S3	
		C_T	E	C_T	E	C_T	E	C_T	E	C_T	E	C_T	E
Cytoplasmic heat shock protein 70	0.5% PB	21.33	2.03	21.24	2.18	21.28	2.05	21.61	2.05	21.69	1.97	21.11	1.97
	Control	28.73	1.94	29.31	1.96	29.14	1.94	29.36	2.03	28.75	2.02	34.81	1.99
NADH dehydrogenase subunit 4	0.5% PB	17.93	2.11	18.33	2.07	19.43	2.16	17.44	2.06	17.27	2.06	16.85	2.11
	Control	29.89	1.93	29.11	2.08	30.74	1.92	30.06	2.08	29.42	2.11	33.94	2.03
Elongation factor 1-alpha	0.5% PB	22.67	2.00	21.92	2.15	23.32	2.06	20.52	2.20	21.88	2.03	21.39	2.15
	Control	28.23	2.05	27.51	1.94	30.39	1.92	28.71	2.03	26.87	1.90	29.92	2.05
Cytoplasmic heat shock protein 70	*E. coli*/ *P. termitis*	24.23	1.91	25.18	2.01	22.36	2.04	27.85	1.92	21.80	2.04	21.26	2.08
	Control	33.79	1.92	33.69	1.91	31.86	1.91	35.01	1.83	33.37	2.02	33.83	2.01
NADH dehydrogenase subunit 4	*E. coli*/ *P. termitis*	18.18	2.07	17.95	1.99	17.52	1.99	21.61	2.12	17.93	2.05	17.72	2.04
	Control	31.62	1.96	32.75	1.90	32.86	1.95	33.69	2.06	33.30	2.02	28.89	1.91
Elongation factor 1-alpha	*E. coli*/ *P. termitis*	24.23	2.01	24.39	2.05	23.21	1.98	28.24	1.92	20.65	2.15	21.57	1.94
	Control	36.77	1.74	34.63	1.83	28.53	2.02	36.26	1.82	30.99	1.96	30.28	1.86

3.3.1 Effects of Colony

Regardless of treatment or caste, R values were not significantly different between the 3 FST colonies. Figure 3.2 shows mean R values, averaged across the 3 colonies, for treated and untreated workers and soldiers for the 8 inducible genes in this study. In order to determine the effects of treatment and/or caste on constitutive and induced expression of target amplicons, it was first necessary to test for variation between biological replicates, i.e., between the 3 FST colonies. Multivariate analyses of variance were performed to test for variance between colonies and for interactions between colony and treatment or between colony and caste. Significance testing showed no effect of colony for these parameters at any of the 10 target genes. Probabilities of colony variability and interaction ranked from 0.1265 to 0.7823 (mean= 0.5925, SD= 0.1939), which were well outside of the range for significant P values (<0.05).

3.3.2 Effects of Treatment

Treatment type had a significant ($P< 0.05$) effect on the expression of each of the 8 target genes that were inducible, relative to the 3 reference genes, in this study. Comparisons of relative expression and tests for differential expression yielded varying levels of constitutive and induced expression for target genes across treatment groups (0.5% PB and *E. coli/ P. termitis)*. For the sake of clarity, the results for inducible target genes are given, from this point further, according to the order in which these genes appear in Table 3.2 above.

Figure 3.2 (A) shows that CYP 15A1 was induced in both types of treatment; however, relative expression was significantly higher ($F= 88.03$, $P< 0.0001$: PROC GLM) for the *E. coli/ P. termitis* treatment ($R= 5.373 \pm 3.940$) as compared to the 0.5% PB treatment ($R= 3.125 \pm 0.867$). Similarly, relative expression of MatE 1 (Fig. 3.2-B) was significantly higher ($F= 32.37$, $P<0.001$) in the *E. coli/ P. termitis* treatment group ($R= 2.287 \pm 2.218$).

Induced expression of GST (R= 0.718 ± 0.693) was significantly higher (F=3.53, P= 0.0312) for the 0.5% PB treatment than for the *E. coli/ P. termitis* treatment. The Gram-negative bacterial binding protein (GNBP 2) showed induced expression in both treatment types (Fig 3.2-D); however, relative expression of GNBP 2 was significantly higher (F= 28.08, P< 0.0001) for termites injected with *E. coli/ P. termitis* (R= 1.136 ± 1.164) than for termites fed with 0.5% PB (R= 0.862 ± 0.892). Also induced in both treatment groups was the Chitin binding protein (CBP: Fig 3.2-E), which was significantly more induced (F= 31.19, P< 0.0001) in *E. coli/P. termitis* injected termites (R= 8.082 ± 2.199) than in 0.5% PB fed termites (R= 2.501 ± 1.125).

The lipopolysaccharide-binding protein (LBP) was induced in both treatments, while Lectin C and Ficolin 2 were only induced in the 0.5% PB treatment (Fig. 3.2-F-H). Specifically, relative expression (R= 3.700 ± 2.604) of LBP (Fig. 3.2-F) was significantly higher (F= 33.03, P< 0.001) in the 0.5% PB treatment group, and relative expression of Lectin C (Fig. 3.2-G) and Ficolin 2 (Fig. 3.2-H) was significantly higher (F= 61.72, P< 0.0001 and F= 23.78, P<0.001, respectively) for termites fed with 0.5% PB than for termites injected with *E. coli/ P. termitis* (R= 1.87 ± 2.794 and R= 0.611 ± 0.606, respectively).

3.3.3 Effects of Caste

Tests for differential expression showed that caste (worker or soldier) had a significant (P< 0.05) effect on levels of expression in 6 of the 8 inducible target genes (CYP 15A1, MatE 1, GST, GNBP 2, CBP and Lectin C, Fig. 3.2-A-E, G). CYP 15A1 was induced in both workers (R= 5.952 ± 3.356) and soldiers (R= 2.546 ± 0.996), with a significant interaction (P= 0.0315) of treatment and caste, due to the significantly higher (F= 34.79, P< 0.0001) expression of CYP 15A1 in workers as compared to in soldiers (Fig 3.2-A).

Table 3.4: Mean R Values and Standard Deviations for Target Gene Amplicons**.** R Values and Standard Deviations (SD) values were averaged across 5 technical replicates and are listed for each reference gene transcript, treatment type, caste and colony. W1, W2, W3 = workers, and S1, S2, S3 = soldiers from Colony 1, 2 and 3, respectively. Data showing induced target gene expression (significantly increased R in treated vs. untreated termites) are highlighted in grey.

GENE	TREATMENT	W1		W2		W3		MEAN		S1		S2		S3		MEAN	
		R	SD	R	SD	R	SD	R	SD	R	SD	R	SD	R	SD	R	SD
CYP 15A1	0.5% PB	1.04	0.64	1.56	0.95	5.30	2.85	2.63	1.20	4.98	3.21	3.06	1.71	2.81	1.45	3.62	1.18
	Control	0.11	0.16	0.10	0.16	0.00	0.00	0.07	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CYP 314A1	0.5% PB	0.02	0.00	0.03	0.02	0.01	0.01	0.02	0.01	0.01	0.00	0.02	0.00	0.00	0.00	0.01	0.01
	Control	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.17	0.29
MatE 1	0.5% PB	0.03	0.08	0.00	0.00	0.16	0.14	0.07	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.01	0.02	0.51	0.02	0.18	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.00
GST	0.5% PB	1.35	0.98	2.88	2.68	0.07	0.04	1.43	1.34	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
DSCAM Ig7A	0.5% PB	0.02	0.01	0.01	0.02	0.04	0.02	0.02	0.01	0.03	0.01	0.08	0.04	0.01	0.01	0.04	0.02
	Control	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
GNBP 2	0.5% PB	2.87	2.31	1.75	1.32	0.37	0.27	1.66	1.02	0.00	0.00	0.02	0.01	0.16	0.29	0.06	0.09
	Control	0.04	0.07	0.00	0.00	0.00	0.00	0.01	0.04	0.01	0.01	0.03	0.07	0.03	0.07	0.02	0.01
CBP	0.5% PB	4.98	6.18	3.06	2.56	0.97	0.46	3.00	2.90	5.24	4.62	0.63	0.88	0.14	0.11	2.00	2.42
	Control	0.00	0.00	0.01	0.03	0.01	0.03	0.01	0.02	0.02	0.02	0.01	0.03	0.01	0.01	0.01	0.01
LBP	0.5% PB	13.65	5.75	0.24	0.32	0.24	0.10	4.71	3.20	3.14	1.13	4.64	3.17	0.30	0.29	2.69	1.48
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lectin C	0.5% PB	0.03	0.02	0.06	0.10	6.22	1.38	2.10	0.77	2.12	0.97	10.05	5.76	3.33	1.60	5.17	2.60
	Control	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.00
Ficolin 2	0.5% PB	0.05	0.02	2.00	2.89	2.38	1.23	1.48	1.44	2.17	1.06	2.51	0.97	0.10	0.15	1.60	0.50
	Control	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01
CYP 15A1	*E. coli/ P. termitis*	4.54	3.37	6.51	2.33	16.77	8.59	9.27	3.35	3.85	2.65	0.45	0.25	0.13	0.16	1.48	2.06
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.00
CYP 314A1	*E. coli/ P. termitis*	0.02	0.01	0.00	0.00	0.05	0.03	0.02	0.01	0.33	0.11	0.01	0.00	0.02	0.02	0.12	0.18
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MatE 1	*E. coli/ P. termitis*	8.72	4.62	3.72	4.13	1.06	0.95	4.50	1.99	0.04	0.05	0.00	0.00	0.18	0.29	0.08	0.10
	Control	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.00
GST	*E. coli/ P. termitis*	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DSCAM Ig7A	*E. coli/ P. termitis*	0.00	0.00	0.00	0.00	0.09	0.05	0.00	0.01	0.15	0.11	0.02	0.01	0.15	0.10	0.11	0.05
	Control	0.01	0.01	0.00	0.01	0.01	0.01	0.07	0.09	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01
GNBP 2	*E. coli/ P. termitis*	2.45	1.98	2.65	2.12	1.71	1.15	2.27	0.52	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00
	Control	0.00	0.01	0.04	0.07	0.00	0.00	0.01	0.04	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.00
CBP	*E. coli/ P. termitis*	11.37	0.37	5.38	8.86	5.68	4.92	7.47	4.25	11.28	6.03	1.84	2.83	12.95	4.37	8.69	1.60
	Control	0.00	0.00	0.02	0.03	0.00	0.00	0.01	0.01	0.01	0.02	0.00	0.00	0.01	0.01	0.01	0.01
LBP	*E. coli/ P. termitis*	8.40	0.51	1.26	0.66	0.14	0.05	0.07	0.09	0.67	2.36	0.79	0.62	0.61	0.97	0.69	0.92
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lectin C	*E. coli/ P. termitis*	0.82	0.45	0.54	0.26	1.25	0.78	0.87	0.26	6.60	5.57	0.36	0.35	1.65	1.24	2.87	2.79
	Control	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.00
Ficolin 2	*E. coli/ P. termitis*	0.28	0.20	0.22	0.32	2.58	1.30	1.02	0.61	0.41	0.14	0.01	0.01	0.17	0.13	0.20	0.07
	Control	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01

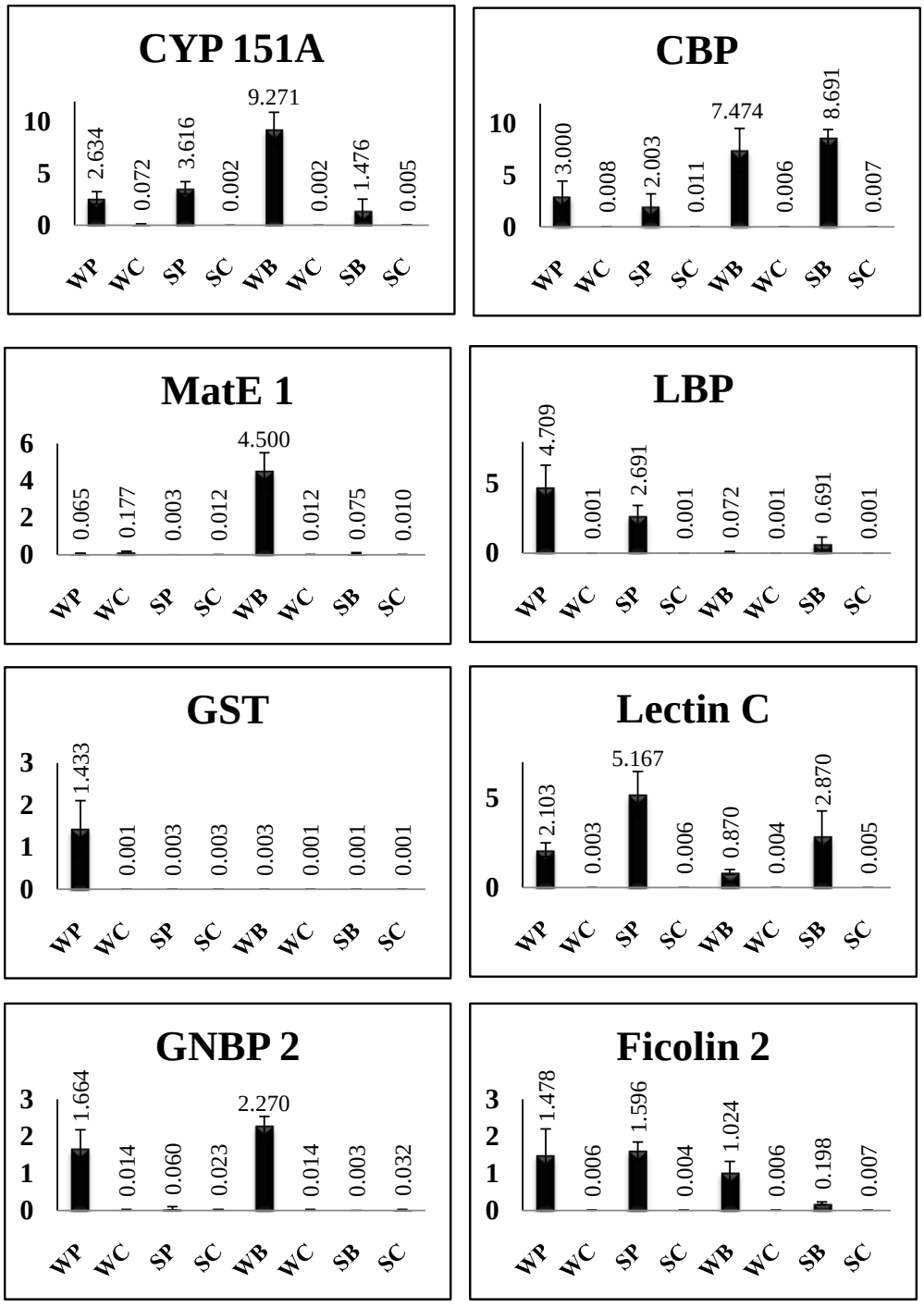

Figure 3.2: Constitutive and induced expression of target gene amplicons. The y-axes denote relative expression in R units (note the range of R varies across graphs). The x-axes denote caste (W= worker, S= soldier) and treatment type (P= 0.5% PB, B= *E. coli/ P. termitis* and C= control R values are averaged across Colonies 1, 2 and 3. No induced expression was observed for CYP 314A1 or DSCAM IG7A (not shown).

Constitutive expression of CYP 15A1 (Fig 3.2-A: R= 0.072 ± 0.091) was also significantly increased (F= 213.65, P< 0.001, PROC GLM) for untreated workers as compared to untreated soldiers. Constitutive expression of MatE 1 (Fig. 3.2-B) was significantly higher (F= 40.16, P< 0.0001) in untreated workers (R= 0.177 ± 0.01) than in untreated soldiers (R= 0.011 ± 0.003) as well. MatE 1 was not induced in FST soldiers but was instead induced only in workers (R= 2.283 ± 2.279). Similarly, GST (Fig. 3.2-C) was only induced in workers (R= 0.718 ± 0.639). In fact, the only detectable level of induced GST expression in this study was found in workers fed with 0.5% PB. The Gram-negative bacterial binding protein (GNBP 2: Fig. 3.2-D) was, similarly, only inducible in workers (R= 1.967 ± 0.466), with a highly significant increase in relative expression (F= 121.23, P< 0.001) as compared to that of soldiers (R= 0.0315 ± 0.087).

Similar to what was found in CYP 15A1 (Fig. 3.2-A), CBP (Fig. 3.2-E) was inducible both in workers (R= 5.237 ± 4.249) and in soldiers (R= 5.347 ± 2.415). However, induced expression of CBP was significantly higher for workers (F= 8.10, P< 0.05) than for soldiers. LBP (Fig 3.2-F) was also induced in both castes (workers: R= 3.98 ± 2.856, soldiers: R= 1.691 ± 1.288), with no significant difference in expression of this target gene transcript as an effect of caste (F= 0.16, P= 0.692). There was, however, an effect of caste on the inducible expression of Lectin C (Fig 3.2-G), which was the only target gene that showed significantly higher (F= 47.53, P< 0.001) expression in soldiers (R= 4.018 ± 1.189) than in workers (R= 1.486 ± 1.183). Finally, Ficolin 2 (Fig 3.2-H) was induced both in workers (R= 1.251 ± 1.593) and in soldiers (R= 0.897 ± 1.056), with significance in the favor of the worker caste (F= 7.31, P< 0.05).

Chapter 4: Discussion

In this study primers were developed and used, in conjunction with primers previously used in the FST by Husseneder et al. (2012), to study 10 target genes involved in major innate immunity pathways and/or detoxification via xenobiotic metabolism in FST. Genes putatively involved in Toll pathway signaling included a gram-negative binding protein (GNBP 2: Kim et al. 2000), a chitin binding protein (CBP), 2 lectin-like genes (LBP and Lectin C: Fujita 2002, Yu et al. 2002) and a ficolin precursor (Ficolin 2: Middha and Wang 2008, Zhou et al. 2011). Most likely to be involved in the Imd pathway was a gene that expresses a hemolymph lipopolysaccharide binding protein (LBP). Also investigated were two genes (CYP 151A and CYP 314A1) putatively associated with phase I modification during detoxification (i.e., oxidation, hydrolysis and/or reduction: Sun et al. 2006, Willoughby et al. 2007), a gene encoding phase II conjugation enzymes (GST: Lumjuan et al. 2007, Ramzi et al. 2009, Ranson and Hemingway 2005, Sun et al. 2006, Willoughby et al. 2007), a Down Syndrome cell adhesion molecule (DSCAM Ig7A) and a gene encoding a putative phase III transmembrane protein (MatE 1: Kaatz et al. 2005).

Termites were experimentally infected (via septic injury) based on a modification of published methods used to induce immunity-related gene expression in termites (Calleri et al. 2006). To induce target genes putatively associated with the Toll and/or Imd immunity pathways, and to increase the likelihood that a broad array of target genes would be up-regulated, FST workers and soldiers were infected with both gram-negative (Imd- pathway) and gram positive (Toll- pathway) bacteria. Placebo injections were not performed on control termites, since there was no need to separate between injury and infection related genes (because injury and infection usually occur simultaneously in nature, and the combined array of gene expression involved in

septic injury was to be investigated). The 24 hr. sublethal exposure time was consistent with successful induction of innate immunity in insects (i.e., peptide synthesis in *D. melanogaster:* Fehlbaum et al. 1994 and in *P. spiniger:* Lamberty et al. 2001). The 0.5% PB treatment was used to induce transcription of genes associated with detoxification via xenobiotic metabolism in FST workers and soldiers. PB was the chosen inducer (rather than pyrethroid, for example) because it is non-repellent, does not require resistant organisms and has long been used to induce a broad range of metabolic enzymes in insects (Yu and Terriere 1971, Ottea et al. 1981, Feyereisen 2005, Le Goff et al. 2006, Sun et al. 2006). The concentration of PB fed to workers and soldiers (0.5%) in this study was consistent with those of previous studies: from 0.5% in *Blattella* (Brown et al. 2003) to 1% in *M. domestica* (Yu and Terrier 1971), as was the time frame (24 hrs.) used: from 4 hrs. in *D. melanogaster* (Willoughby et al. 2007) to 5 days in cockroaches (Brown et al. 2003). Furthermore, Brown et al. (2003) showed that, of six known xenobiotic inducers investigated (methoxyresorufin, acetone, ethanol, ethoxyresorufin, resorufin, benzyloxyresorufin, phenobarbital, 3-methylcholanthrene, and beta-naphthoflavonon), only PB consistently induced monooxygenase activity in *B. germanica*.

Induced expression was first quantified (via qRT-PCR) and then compared within and between colonies, treatments and castes. Since qRT-PCR is a highly sensitive technique for measuring gene expression, extra care was taken at each point of sample preparation, data collection and statistical analysis to control for experimental variation. For instance, samples were extensively normalized (i.e. before and after RNA extraction and cDNA synthesis). In addition, induced expression was calculated relative to that of 3 reference genes (Thellin et al. 1999, Silver et al. 2006, Infante et al. 2008) accounting for even slightly variable individual reaction efficiencies (Pfaffl 2001). Finally, both normally and non-normally distributed data

points were included in multivariate analyses of variance (via PROC GLM and GLIMMIX, respectively).

There was no differential expression found among FST colonies in this study, regardless of treatment or caste. This result is in contrast to other studies, which have shown that production of immune response proteins (e.g. Lamberty et al. 2001, Traniello et al. 2002, Rosengaus et al. 2007) and metabolic enzymes associated with detoxification (e.g. Valles and Woodson 2002) varies among termite colonies. However, these studies differ from the present study, either in the inducing agent (e. g. fungal infection via *M. anisopliae*: Traniello et al. 2002, Rosengaus et al. 2007), the method used to measure induction (e.g. HPLC purification: Lamberty et al. 2001, mortality assays: Traniello et al. 2002, Valles and Woodson 2002) or in the species of termite under investigation (e.g. *P. spiniger*: Lamberty et al. 2001, *Z. angusticollis*: Traniello et al. 2002).

The current study measured, quantitatively, the transcriptional up-regulation of genes encoding AMPs and enzymes putatively involved in detoxification via xenobiotic metabolism. All but 2 of the 10 target genes were inducible in FST workers and/or soldiers exposed to either non-pathogenic bacteria or a sublethal dose of PB. The two targets that were not induced (CYP 314A1, DSCAM Ig7A) encode for a CYP P450 monooxygenase and a cell adhesion molecule with immunoglobulin domains, respectively. It is feasible that these genes are simply not inducible with PB or septic injection in *C. formosanus*, but may instead be inducible with agent(s) other than those used in this study. For example, DSCAM molecules are involved in axon guidance and nervous system targeting in *D. melanogaster* (Zhan et al. 2004), while others are associated with defense against *Plasmodium* in *A. gambiae* (Dong et al. 2006).

Expression of each of the 8 induced target genes was significantly influenced by treatment type. The *E. coli/ P. termitis* treatment induced increased expression of CYP 151A, MatE 1, GNBP 2, CBP, LBP, Lectin C and Ficolin 2, while the PB treatment induced increased expression of CYP 151A, GST, GNBP 2, CBP, LBP, Lectin C and Ficolin 2. Among the genes induced by both treatments, relative expression of CYP 151A, MatE 1, GNBP 2 and CBP was significantly higher for the *E. coli/ P. termitis* treatment, while LBP, Lectin C and Ficolin 2 were significantly more expressed following treatment with 0.5% PB. Differential expression of GST and GNBP 2 among treatment types was expected, based on previous studies. For instance, Le Goff et al. (2006) showed that 3 GST genes were significantly induced by PB, while Bulmer (2009) reported that GNBP 2 acts as a PRP, triggering immune response in several termite species (i.e. *Nasutitermes corniger, Zootermopsis augusticollis, Cryptotermes secundus*, and *Reticulitermes flavipes*)**.**

Gram-negative binding proteins recognize bacterial and fungal PAMPs and has beta 1,3 glucanase activity (Bulmer et al. 2009), which activates the Toll pathway. Therefore, it was unsurprising that relative expression of GNBP 2 was significantly higher for termites injected with *E. coli/ P. termitis*. GNBP 2 expression was also significantly higher in workers than in soldiers. When observing differential gene expression among these castes, it is important to note their very different roles in the colony. FST workers forage for and distribute food throughout the colony, build the nest and interconnected tunnels and care for the brood, while soldiers are mainly responsible for defending the colony from invaders (King and Spink 1974, Su and Tamashiro 1987). Therefore, workers have more social interactions and are more likely than soldiers to encounter environmental pathogens and toxins. Furthermore, unlike workers, soldiers do not participate in grooming or nest building behaviors. Soldiers may receive passive

immunization from the GNBP 2 produced by the workers' salivary glands which is incorporated in nest building material (Bulmer et al. 2009). Soldiers may also have antibacterial and/ or antifungal activity via chitin assembly, wound healing and systemic protection related to increased CBP expression, which has been suggested for other highly sclerotized arthropods (Destoumieux et al. 2000). Differential production of immune response AMPs between castes has been previously demonstrated in termites (Rosengaus et al. 1999, Lamberty et al. 2000, Traniello et al. 2002). For example, the EST library of *R. flavipes* (Steller et al. 2010) showed potential biased expression of putative immune response genes among castes, though they did not quantify expression as part of that study.

In total, expression of 6 of the 8 induced target genes (CYP 151A, MatE 1, GST, GNBP 2, CBP and Lectin C) was significantly influenced by caste in the current study. Significantly increased levels of constitutive and/or induced expression were detected for CYP 151A, MatE 1, GST, GNBP 2, CBP and Lectin C in workers, In fact, 3 of the target genes (GNBP 2, MatE 1 and GST) were induced only in the worker caste. Discussed above are possible explanations for increased expression of putative immune response genes in FST workers, and this bias was most visible for the GNBP 2 and MatE 1 genes. It was somewhat unexpected that MatE 1 was induced by the *E. coli/ P. termitis* treatment, as MatE proteins have been described as multi drug resistance (MDR) efflux proteins thought to be involved in detoxification (Kaatz et al. 2005) and function as transmembrane pumps, mediating resistance to antibiotics in bacteria (Plésiat 2007). However, MatE proteins may be involved in disease resistance in plants. Nawrath et al. (2002) investigated a gene (*EDS5*) that encodes for a MatE protein in *Arabidopsis* plants. They found that *EDS5* expression was strongly induced by inoculation with an avirulent strain of *Pseudomonas syringae* and, thus was likely involved in antimicrobial plant defense. Similarly,

it is feasible that the MatE 1 gene is involved in immune response in this species, and this is indicated by the induced expression of MatE 1 in FST workers and soldiers exposed to *E. coli/ P. termitis*.

PB is a barbiturate used to treat epilepsy in humans. PB acts on GABA-gated chloride channels in the central nervous system (similar in activity to some termiticides, i.e. Fipronil: Cole et al. 1993, Ratra and Casida 2001, Boyd et al. 2002) to reduce excitatory synaptic response. GSTs convert PB into conjugates that are less reactive, more water soluble and less toxic (Ramzi et al. 2009, Lumjuan et al. 2007). Exposure to PB has been previously shown to induce GST activity in flies (*M. domestica:* Yu and Terriere 1971, *D. melanogaster:* Sun et al. 2006, Willoughby et al. 2007), *A. aegypti* (Lumjuan et al. 2007) and in two true bugs (*Eurygaster integriceps*, *Brachynema germari:* Ramzi et al. 2009). Therefore, it was unsurprising that relative expression of GST was significantly higher for workers exposed to the 0.5% PB treatment. The fact that GST was only induced in workers shows that expression was again biased in favor of workers. This differential expression may explain the differing levels of insecticide susceptibility previously found in FST workers and soldiers i.e., workers were less susceptible than soldiers to insecticides (Gatti and Henderson 1996, Gatti et al. 2002, Valles and Woodson 2002). However, unlike as in *D. melanogaster* (Sun et al. 2006), the links between susceptibility, gene expression and metabolic enzyme production have not yet been confirmed in FST.

Cytochrome P450s have numerous functions in insects, including not only detoxification of insecticides and xenobiotics (Feyereisen 2005, Terrier 1984), but also regulation of caste development and metabolism of juvenile hormone (JH: Feyereisen 2005, Helvig et al. 2004, Mao et al. 2005, Terrier 1984, Zhou et al. 2006), which is, in turn, influenced by pheromones and/or

colony density in termites (Mao et al. 2005). CYP 151A epoxidizes methyl farnesoate to juvenile hormone III in cockroaches (Helvig et al. 2004) and was found to function similarly in termites (Feyereisen 1999, Sutherland et al. 2000, Feyereisen 2005, Tarver et al. 2010, Tarver et al. 2012). In the current study, CYP 151A was (the only target gene) constitutively expressed in FST workers and soldiers, induced by both treatment types and showed significant increase in expression in workers relative to soldiers. This gene has an important role in JH regulation and biosynthesis (Tarver et al. 2012), which may explain the level of constitutive expression observed in this study. In addition, CYP 151A appears to have a role in immunity and xenobiotic metabolism which warrants further investigation.

Lectins and ficolins are associated with the Lectin complement pathway and, along with fibrinogen-related proteins (FREPs), are known to catalyze innate immune function via the Lectin complement pathway (Middha and Wang 2008, Zhou et al. 2011). In the current study, the LBP, Lectin C and Ficolin 2 genes were induced in both the *E. coli/ P. termitis* treatment and the 0.5% PB treatment. Induced expression of these lectin complements was significantly higher in the PB treated termites, both in workers and in soldiers. The specific links between PB exposure and the Lectin complement pathway are not well understood in insects; however, it is known that PB induces genes involved in carbohydrate metabolism (Sun et al. 2006) and that carbohydrates act as binding sites for lectins and ficolins (Pritchard et al. 1994). Lectins and ficolins, which activate the lectin pathway and enhance phagocytosis and opsonization, (Endo et al. 1996) have numerous binding sites that have been shown to be altered after PB exposure (Pritchard et al. 1994). For example, Middha and Wang (2008) showed that fibrinogen-like (FBG) domains involved in innate immunity were triggered by xenobiotic exposure in 12 *Drosophila* species.

Chapter 5: Significance and Future Research

The FST is an extremely invasive pest species that is responsible for over $1 billion in management and repair costs per year in the U.S. (Culliney and Grace 2000, Pimentel et al. 2005). Chemical treatments for this termite come with risks, such as bioaccumulation, groundwater contamination and non-target effects. Biological control efforts have been fraught with difficulties and have yet to be demonstratively effective in the field (Chouvenc et al. 2011). Therefore, development of treatment tactics that enhance the effectiveness of current chemical or biological control methods is essential. Innate immune response and mechanisms of xenobiotic metabolism are known to influence control efforts (Bechkage, 2008), and the FST is a valuable model for functional genomic investigations into these mechanisms. The termites are regularly exposed to bacteria and xenobiotics where they nest and feed. They live in warm, moist environments, conducive to microbial growth and are exposed to chemically treated soils and building materials.

Structural genomics has elucidated the genetic underpinnings of immune response and detoxification in this species (Husseneder et al. 2012), and the current study employed functional genomics to investigate how expression levels of these genes change dependent upon bacterial or xenobiotic exposure. In this study, I successfully amplified genes putatively associated with both of the major innate immunity signaling pathways (Toll: GNBP 2, CBP Imd: LBP) as well as the Lectin complement pathway (Lectin C and Ficolin 2), a Down Syndrome cell adhesion molecule (DSCAM Ig7) and genes involved in all 3 phases of xenobiotic metabolism (Phase I: CYP 15A1 and CYP 314A1, Phase II: GST, Phase III: MatE 1). Protocols for inducing these genes in FST workers and soldiers were established, as were the reference genes and calculations used to quantify relative expression. This study gives new insight into genes that are up-regulated in FST

during immune or chemical defense. This is the first step towards developing novel avenues of effective, yet environmentally responsible, control tactics. For example, disruption of genes (e.g. via RNA interference) involved in immune response and detoxification may be used to decrease fitness or increase susceptibility in the termites and thus increase efficiency and reduce the amount of insecticides needed for control. Enhancement of biological control efforts may be achieved by inhibiting expression of immune response genes (Bulmer et al. 2009).

Knowing which genes are primarily expressed by workers is significant, since the workers interact with the entire colony (e. g. via feeding and grooming activities) and leave the nest to forage for food. These characteristics make workers the targets for bait delivered insecticides currently in use (Sentricon, Dow AgroSciences). Disruption of FST worker defense mechanisms via inhibition of gene expression (i.e. RNAi: Novina et al. 2004, Zhou et al. 2008, Bautista et al. 2009) may make termites more susceptible to pathogens or insecticides. Understanding the links between gene regulation and gene expression, after exposure to pathogens and/or chemical agents, will enhance management of insect pest populations (Zhu and Snodgrass 2003), and may provide insights into how these pathways are regulated in other species as well.

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Appendix A: Abbreviations and Terminology

Adhesion molecules: Proteins expressed on the surface of cells that mediate binding of immune system cells to other cells.

Alate: Having wings; in FST, the winged reproductives.

AMP: Anti-microbial protein.

Antigen: A substance that, when introduced to a host, is recognized by the host immune system as foreign and effectively elicits immune responses. The structure on an antigen molecule that interacts with the combining site of an antibody is an epitope.

Bioaccumulation: Capacity of a chemical to be stored in fatty tissue from environmental uptake.

cDNA: Complementary DNA: a DNA molecule that is complementary to a specific messenger RNA, synthesized from an RNA template or complex sample of mRNAs using reverse transcriptase.

Coagulation: Part of hemeostasis (the cessation of blood loss from a damaged vessel), wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel.

Constitutive expression: Genes that are transcribed continually compared to a facultative gene that is only transcribed as needed.

Cytochrome P450s: Heme-containing proteins that absorb light maximally at 450nm when carbon monoxide binds to the proteins. Cyp P450s rank first among phase I biotransforming enzymes for its catalytic versatility and the number of xenobiotics it detoxifies or activates to reactive intermediates.

Dose/ dosage: dose refers to exact amount of compound administered (i.e. topical or injection bioassay), whereas dosage refers to amount of compound that is present in an organism's environment (i.e. contact bioassay).

Encapsulation: Confinement of an individual molecule within a larger molecule.

EPA: Environmental Protection Agency.

EST library: Expressed sequence tags are sites derived from cDNAs. These are compared with known sequences via BLAST and then annotated for the target species to create the library.

FST: Formosan subterranean termite, *Coptotermes formosanus* (Shiraki).

Gene expression: Conversion of information from the gene into mRNA via transcription and then to protein via translation resulting in the phenotypic manifestation of the gene. Gene expression of the non-protein coding genes, such as the rRNA and tRNA genes, involves only transcription and not translation.

Genome: The sum total of genes in a cell.

GNBP: Gram-negative binding protein.

Housekeeping gene: Housekeeping genes are those genes that are always expressed because they code for proteins that are constantly required by the cell, hence, they are essential to a cell and always present under any conditions. It is assumed that their expression is unaffected by experimental conditions.

Immunity: The condition of being immune, the protection against infectious disease conferred either by the immune response generated by immunization or previous infection or by other non-immunologic factors.

Induced/ inducible: To initiate or increase the production of (an enzyme or other protein) at the level of genetic transcription; to cause an increase in the transcription of the RNA of (a gene).

Mannose-binding lectin pathway: A pathway of the complement system that is independent of specific antibody and activated by foreign carbohydrates including mannose and N-acetyl glucosamine.

Mixed model analysis of variance: In statistics, analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables. Mixed models describe situations where both fixed and random effects are present.

mRNA: Messenger RNA are transcribed and translated from amino acid sequence to protein.

Nymph: Immature forms that resemble the adult, apart from having underdeveloped reproductive organs and/or wings, and develop into the adult without a pupal stage.

PAMP: Pathogen Associated Molecular Pattern.

Pathogens: Infectious agents that cause disease to its host.

PB: Phenobarbital.

PCR: Polymerase chain reaction, a technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase.

PGRP: Peptidoglycan Recognition Protein.

Phagocytosis: Engulfing of microorganisms or other cells and foreign particles by phagocytes.

Pheromone: chemical released by an organism that has a behavioral (releaser pheromone) or physiological (primer pheromone) effect on another individual of the same species.

PRP: Pattern Recognition Protein.

qRT-PCR: Quantitative real time polymerase chain reaction, based on the polymerase chain reaction, that is used to amplify and simultaneously quantify a targeted DNA molecule. PCR product is monitored cycle-by-cycle by combining thermal cycling, fluorescence detection and application-specific software.

RNA: Ribonucleic acid, biomolecule that has an informational, structural and enzymatic role. The structure is of ribose units joined in the 3' and 5' positions through a phosphodiester linkage with a purine or pyrimidine base attached to the primary position.

RNAi: Mechanism for regulating gene expression by interfering with gene transcription and silencing genes.

SYBR Green: In real-time PCR detection, this dye binds to double-stranded DNA and upon excitation emits light. The dye cannot distinguish between specific and non-specific DNA products.

Toxic/ toxicity: Pertaining to, due to or of the nature of a poison or toxin, manifesting the symptoms of severe infection.

Toxin: A poison or poisonous agent. A toxin is a biological agent, as opposed to a toxicant, that is man-made.

Transcription: Process of constructing a messenger RNA molecule using a DNA molecule as a template, with resulting transfer of genetic information to the messenger RNA.

Virulence: The degree of pathogenicity of a microorganism, as indicated by invasiveness and mortality.

Xenobiotic compound: Compounds foreign to the body or living organisms, specifically chemical compounds, i.e. pesticides.

Appendix B: Comprehensive Table of Primers Designed and Tested for Reference Genes

GENE	FUNCTION	ACCESSION NO.	PRIMER	SEQUENCE (5'-3')	Tm	GC%
Cytoplasmic heat shock protein 70	ATP and nucleotide binding	FK835495	HSP-1F	AGCCTTGGCCACAACAGTGCAA	59.7	55
			HSP-1R	GGAGCAGGAGCAGGACCGACT	59.9	67
			HSP-2F	CAAGCCTTGGCCACAACAGTGC	59.4	59
			HSP-2R	GGAGCAGGAGCAGGACCGACT	59.9	67
NADH dehydrogenase subunit 4	Mitochondrial electron, sodium ion and proton transport, ubiquinone activity	FK833785	NADH-1F	ACGAAGCAACCCATAACCACCAAGC	59.9	52
			NADH-1R	GGGCTCATGTTGAGGCTCCTGTT	58.9	56
			NADH-2F	ACGAAGCAACCCATAACCACCAAGC	59.9	52
			NADH-2R	AGGGCTCATGTTGAGGCTCCTGTT	59.8	54
Elongation factor 1-alpha	GTP binding, regulation of tranlational elongation	FK834645	EFA-1F	GCAGCCACTTGGAAAGAAAG	54.5	50
			EFA-1R	TGAGAAGGCCCAGAAGAAGA	55.5	50
			EFA-2F	GGCGGTCACATTTCTCCTTA	54.8	50
			EFA-2R	CTGAACCACCCTGGTCAGAT	56.6	55
			EFA-3F	GGCGGTCACATTTCTCCTTA	54.8	50
			EFA-3R	GTCACACAGCTCACATCGCT	57.7	55
			EFA-4F	ACAGTCTGCCTCATGTCACG	57.2	55
			EFA-4R	TATCAAGTCTGGTGATGCGG	54.5	50
Glyceraldehyde-3-phosphate dehydrogenase	Dehydrogenase binding, glycolysis	FK834351	GL3PH-1F	CCGCGCACGACGCAAATCAG	59.9	65
			GL3PH-1R	CGAACGACCAGCAGGTGGCA	59.6	65
			GL3PH-2F	TCACGGGCGCAAGGCAGTTC	60.3	65
			GL3PH-2R	GAAGCCGGAGACGCAGCACA	59.7	65
			GL3PH-3F	TCTTCGTCACGGGCGCAAGG	59.7	65
			GL3PH-3R	GAAGCCGGAGACGCAGCACA	59.7	65
TATA-binding protein associated factor 172/ 89B Helicase	Helicase activity, nucleic acid and ATP binding	FK835420	TATA-1F	GTTCAGACCGAGCCCGCCAA	59.4	65
			TATA-1R	GGCATGATGGGGGTGACAGCG	60.1	67
			TATA-2F	TTCAGACCGAGCCCGCCAAC	59.4	65
			TATA-2R	GGCATGATGGGGGTGACAGCG	60.1	67
Ubiquitin specific peptidase 2	Electron carrier and receptor activity, cell matrix adhesion, integrin mediated signalling pathway	FK831079	UBIQ-1F	GCAGGTTTCCGCGCTTTCGC	60.1	65
			UBIQ-1R	CGACTCGATCGTCCGCAGCA	59.3	65
			UBIQ-2F	TTTCCGCGCTTTCGCACCGA	60.3	60
			UBIQ-2R	ACGACTCGATCGTCCGCAGC	59.3	65

Vita

Dawn Simms is currently a Research Associate at Louisiana State University, Department of Pathobiological Sciences, where she began her work in 2012. Previously, she held a nine year appointment as Research Associate and Laboratory Manager at Louisiana State University Agricultural Center, Department of Entomology. During this time, Ms. Simms also served as Molecular Lab Coordinator for the Operation Fullstop Program in New Orleans, Louisiana. As part of this work, she used molecular markers to: 1) identify FST colony boundaries and breeding structures, 2) correlate inbreeding to FST worker size, 3) correlate inbreeding to sexual selection and mating in FST alates, 4) investigate invasion patterns of FST from native and introduced populations, 5) assign FST alates to their colonies of origin and 6) assess treatment success of FSTs. Ms. Simms has extensive laboratory experience in micro- and molecular biology, as well as having a strong educational background in genetics, conservation, entomology and biodiversity. Her research has included mitochondrial and genomic DNA sequencing, microsatellite genotyping, population genetics, gene expression and bioinformatics. To date, Ms. Simms has authored or co-authored 7 publications and 19 presentations on population genetics, invasion biology, partner selection, flight behavior and reproduction in Formosan subterranean termites.